

# FOLIAR UPTAKE OF ATMOSPHERIC REACTIVE NITROGEN POLLUTION

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# FOLIAR UPTAKE OF ATMOSPHERIC REACTIVE NITROGEN POLLUTION

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Several studies have established that gaseous reactive N is capable of being incorporated directly into the leaves and assimilated into plant metabolism. However, no studies have directly quantified the magnitude of foliar N uptake at realistic atmospheric N concentrations or how this entry pathway may influence vegetative responses to increasing atmospheric N deposition. This body of work expands on previous research by explicitly quantifying the direct foliar uptake of atmospheric nitrogen dioxide (NO<sub>2</sub>) using foliar  $\delta^{15}\text{N}$  and the mechanisms driving the capacity for species to incorporate atmospheric reactive N directly through the leaves into biomass over multiple scales in laboratory, mesocosm, and natural systems.

In chapter one, I use foliar  $\delta^{15}\text{N}$  as a tool to quantify the magnitude of foliar NO<sub>2</sub> uptake in tomato (*Lycopersicon esculentum*) and tobacco (*Nicotiana tabacum*). This chapter establishes that gaseous <sup>15</sup>NO<sub>2</sub>-N may be directly incorporated into the leaves of vegetation and is assimilated as a nutritional source under N-limited conditions. In chapter two, I examine a suite of factors including morphological, stable isotope, and nitrate reductase activity in *L. esculentum* and *N. tabacum* to quantify the amount of N incorporated via foliar <sup>15</sup>NO<sub>2</sub> uptake, the variation in foliar uptake capacity between species, and determine whether realistic concentrations of <sup>15</sup>NO<sub>2</sub> influence plant N metabolism. This chapter provides evidence that the magnitude of foliar NO<sub>2</sub>

uptake has the potential to influence plant metabolism and that variation exists among species in the capacity to incorporate  $\text{NO}_2$  via the foliar uptake pathway. In chapter three, I investigate the roles of different root N sources ( $\text{NO}_3^-$  and  $\text{NH}_4^+$ ) on enzyme expression and foliar uptake capacity of  $^{15}\text{NO}_2$  in *N. tabacum*. This chapter contributes to the mechanistic understanding of foliar uptake capacity by integrating relationships among several potential drivers of the foliar uptake pathway. Chapter four examines the variation in natural abundance foliar  $\delta^{15}\text{N}$  patterns and uses these measurements to estimate the importance of soil  $\delta^{15}\text{N}$ , mycorrhizal associations, and foliar N uptake on foliar  $\delta^{15}\text{N}$  patterns. This chapter supports the use of natural abundance foliar  $\delta^{15}\text{N}$  to detect the potential for foliar N uptake in several tree species in temperate forests.

## BIOGRAPHICAL SKETCH

Dena Vallano was born on 9 February 1981 in Greensburg, Pennsylvania.

Dena grew up spending much of her childhood playing in the fields behind her house until they were plowed over to make room for a subdivision. During this time, she fell in love with nature and basketball, never daring to choose between the two.

In 1999, Dena traveled across the state to Philadelphia to pursue her BS in Honors Biology at Villanova University. She began her studies with aspirations of becoming a veterinarian, shifted her focus to bioethics, and eventually took a course in Introductory Ecology that reignited her childhood love and passion for learning about nature. During her sophomore year, she received a Howard Hughes Medical Institute Fellowship to work in the lab of Dr. Joseph Orkiszewski. This experience completely changed her life and introduced her to the wonderful world of laboratory research and plant physiology. While at Villanova, she spent a semester abroad in Melbourne, Australia, an experience that cemented her passion for international travel and further reinforced her desire to study ecosystem ecology. Dena eventually completed her senior honors thesis research examining the effects of the plant hormones kinetin and gibberillic acid on shoot apical meristem development in corn. The mentorship and friendship Dena received while working for Dr. Orkiszewski was the inspiration behind her decision to pursue graduate studies.

She decided to combine her love for plant physiology and ecology and pursue a graduate degree in Ecology and Evolutionary Biology at Cornell University. She joined the lab of Dr. Jed Sparks and began her journey into the

fascinating underworld of direct leaf incorporation of atmospheric reactive nitrogen, which became the topic of her doctoral dissertation. While at Cornell, she became immersed in the field of stable isotope ecology and pretty handy along the way. When not working on her project, Dena had opportunities to work in many amazing locations (Trinidad, Hawaii, etc.) in fields other than her own, which kept her sane and broadened her scientific horizons. She also participated in several outreach and educational opportunities which were incredibly rewarding and memorable experiences.

Since defending her dissertation, Dena has moved to Santa Cruz for a post-doctoral position with Dr. Erika Zavaleta at the University of California Santa Cruz.

To my mother, Linda.

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## PREFACE

Nitrogen (N) is central to the molecular structures of proteins, nucleic acids, and enzymes that regulate metabolic processes in all living organisms. Traditionally, plants are thought to exclusively access reactive N via root uptake of ammonium ( $\text{NH}_4^+$ ), nitrate ( $\text{NO}_3^-$ ), and dissolved organic N from the soil pool. Most of the incorporated reactive N is assimilated in the roots and transported to the shoots in the form of amino acids. These compounds are used in a variety of metabolic processes within the plant that are necessary for nutrition, growth, and photosynthesis. And, in many terrestrial ecosystems, reactive N is often the critical element that limits plant growth. Therefore, the relevance of atmospheric reactive N deposition in ecosystems became increasingly pivotal during the latter half of the 20<sup>th</sup> century and continues to drive research in the fields of plant ecophysiology, biogeochemistry, and stable isotope ecology (Galloway *et al.* 2004, Hill 1971, Nussbaum *et al.* 1999).

The dramatic increase in atmospheric concentrations of reactive N oxides ( $\text{NO}_y$ ) from human activities (car exhausts, factory emissions, etc.) has rapidly increased the amount of reactive N deposition to vegetation (Vitousek 1994). Subsequently, the traditional view that plants incorporate reactive N exclusively through the root uptake pathway was further examined, as this additional N source of atmospheric reactive N became increasingly available to plants. It was discovered that these pollutant compounds were, in fact, being deposited and eventually assimilated into vegetation via *two* inter-related pathways: directly via foliar uptake and indirectly via the root uptake



pathway (Gessler *et al.* 2002, Hanson & Lindberg 1991). In the foliar uptake pathway, gaseous reactive N compounds enter the leaf through the stomata, are rapidly dissolved in the apoplast, and are eventually metabolized. Thus, it is intriguing to ask how this uptake pathway impacts plant physiological processes through influences on growth, photosynthesis, and the plant N budget and further examine the subsequent potential implications on the structure, function and composition of terrestrial ecosystems.

Nitrogen isotope ratios ( $^{15}\text{N}:^{14}\text{N}$ ) became a practical tool for scientists to investigate multiple N sources to plant metabolism, estimate the amount of atmospheric N compounds in an ecosystem, and identify the roles of the various uptake pathways into a plant (Robinson 2001, Robinson *et al.* 1998). Stable nitrogen isotopes are especially useful for these applications because the isotopic composition ( $\delta^{15}\text{N}$ ) of pollutant compounds may considerably differ from the natural N  $\delta^{15}\text{N}$  in the soil. For instance, if the leaf incorporated N source has a significantly different  $\delta^{15}\text{N}$  compared to the root incorporated N source, then the foliar  $\delta^{15}\text{N}$  value will represent both sources and can be used to quantify the proportion of each N source assimilated into plant metabolism. Therefore, the importance of various N sources for plant metabolism could be determined at the leaf level (Ammann *et al.* 1999, Muller *et al.* 1996). Researchers began to use stable isotope techniques to investigate the linkage between gaseous N deposition and N cycling in plants and as a tool to trace the movement and pools of pollutant N compounds within plants (Dawson *et al.* 2002).

Several studies established that gaseous reactive N was capable of being

incorporated directly into the leaves and assimilated into plant metabolism (Boyce *et al.* 1996, Okano *et al.* 1988, Siegwolf *et al.* 2001, Wellburn 1990). Additional work yielded limited insight to the variation in gaseous reactive N assimilation existing across and within species (Gessler *et al.* 1998, Morikawa *et al.* 1998, Sparks *et al.* 2003) and the processes that potentially govern the ability to incorporate reactive N directly through the leaves (Eller & Sparks 2006, Ramge *et al.* 1993). However, to date, few studies have successfully used this tool to examine the magnitude of leaf N uptake at realistic atmospheric N concentrations or how this entry pathway may influence vegetative responses to increasing human-caused N deposition. Further, the mechanisms influencing species variation in the capacity to directly assimilate reactive N via the foliar uptake pathway have not been rigorously examined.

This body of work expands on previous research by explicitly quantifying the direct foliar uptake of atmospheric nitrogen dioxide (NO<sub>2</sub>; a common gaseous pollutant) using foliar  $\delta^{15}\text{N}$  measurements and the mechanisms driving the capacity for various species to incorporate atmospheric reactive N pollutants directly through the leaves into biomass over multiple scales in laboratory, mesocosm, and natural systems.

Chapter one examines the use of foliar  $\delta^{15}\text{N}$  values as a tool to quantify the magnitude of foliar NO<sub>2</sub> uptake and the potential role of the foliar N uptake pathway in N metabolic processes in two herbaceous species, tomato (*Lycopersicon esculentum*) and tobacco (*Nicotiana tabacum*). Using a custom-designed hydroponics-fumigation system, both species were exposed to various concentrations of enriched gaseous <sup>15</sup>NO<sub>2</sub> to the shoot systems and

$\text{NO}_3^-$  to the root systems. Exposure to and assimilation of  $^{15}\text{NO}_2$  into plant tissue stimulated whole-plant biomass production in both species. Both species were capable of incorporating up to 15% of the total assimilated N into biomass directly via the foliar uptake pathway. No significant variation in foliar uptake capacity was observed between species at the whole-plant level. This chapter establishes that gaseous  $^{15}\text{NO}_2\text{-N}$  may be directly incorporated into the leaves of vegetation, assimilated as a nutritional source under N-limited conditions, and may be successfully quantified using foliar  $\delta^{15}\text{N}$  measurements in a laboratory setting.

Chapter two examines a suite of factors including morphological, stable isotope, and nitrate reductase activity in *N. tabacum* and *L. esculentum* to quantify the amount of N incorporated via foliar  $^{15}\text{NO}_2$  uptake, the variation in foliar uptake capacity between species, and determine whether realistic concentrations of  $^{15}\text{NO}_2$  influence plant N metabolism. *N. tabacum* and *L. esculentum* assimilated 15 and 11%, respectively, of  $^{15}\text{NO}_2\text{-N}$  into total biomass via the foliar uptake pathway under low  $\text{NO}_3^-$  supply. Neither *L. esculentum* nor *N. tabacum* linearly increased total biomass accumulation (in g DW) in response to  $\text{NO}_2$  exposure. Both species exhibited differences in the ratio of root to shoot (stem + leaf) tissue in response to differences in root  $\text{NO}_3^-$  supply. There were no strong effects on leaf  $\delta^{13}\text{C}$  values, or leaf and root nitrate reductase activity (NRA) in response to  $^{15}\text{NO}_2$  exposure in either species. Despite only small amounts of variation in NRA, a positive relationship existed between leaf NRA and the amount of  $\text{NO}_2\text{-N}$  incorporated via the foliar uptake pathway in both *L. esculentum* and *N. tabacum*. This chapter provides further evidence that foliar uptake is a significant pathway of

NO<sub>2</sub> entry, the magnitude of foliar NO<sub>2</sub> uptake has the potential to influence plant metabolism, and that some variation exists among species in the capacity to incorporate NO<sub>2</sub> via the foliar uptake pathway.

Chapter three investigates the roles of different root N sources (NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup>) on enzyme expression and the capacity for foliar uptake of gaseous enriched <sup>15</sup>NO<sub>2</sub> in *N. tabacum*. The proportion and amount of NO<sub>2</sub>-N incorporated into biomass via direct foliar uptake was not influenced by differences in the root N source, suggesting that the overall rate of foliar NO<sub>2</sub> uptake is not strongly impacted by the form of N supplied to the root system in *N. tabacum*. When exposed to gaseous NO<sub>2</sub>, plants grown solely on NO<sub>3</sub><sup>-</sup> or NH<sub>4</sub><sup>+</sup> incorporated 12.0 ± 0.6 and 12.4 ± 0.6% of total plant N through foliar uptake, respectively. *N. tabacum* did not increase total biomass accumulation (in g DW) in response to NO<sub>2</sub> exposure, regardless of root N supply. Fumigated plants grown under NO<sub>3</sub><sup>-</sup> supply showed a decrease in the ratio of root to shoot (stem + leaf) tissue compared to non-fumigated plants, but this result was not observed in fumigated plants grown under NH<sub>4</sub><sup>+</sup> supply. Interestingly, we found altered enzyme activities associated with availability of different root N sources and NO<sub>2</sub> exposure. Overall, leaf NR activity decreased and leaf GS activity increased in plants exposed to NO<sub>2</sub>, regardless of the root N source. Results suggest that plant performance and foliar uptake capacity are independent of the root N form and that enzyme expression within the primary N reduction pathways may be correlated with foliar uptake capacity in *N. tabacum*. This chapter contributes to the mechanistic understanding of direct foliar uptake capacity by integrating relationships among several potential drivers of the foliar uptake pathway.

Chapter four examines the variation in natural abundance foliar  $\delta^{15}\text{N}$  patterns among several dominant hardwood tree species and uses these measurements to estimate the relative importance of soil  $\delta^{15}\text{N}$ , mycorrhizal associations, and foliar N uptake on foliar  $\delta^{15}\text{N}$  patterns in response to different levels of atmospheric reactive N deposition in temperate forest ecosystems. Using a potted plant mesocosm system, the relationship between foliar  $\delta^{15}\text{N}$ , soil  $\delta^{15}\text{N}$  and ambient atmospheric N concentration was determined while controlling for mycorrhizal associations in red maple (*Acer rubrum*) seedlings. Along the same temporal and spatial scales, foliar and soil  $\delta^{15}\text{N}$  patterns were determined in adult stands of several dominant hardwood species. The potential influence of direct foliar N uptake, soil  $\delta^{15}\text{N}$ , and mycorrhizae on foliar  $\delta^{15}\text{N}$  patterns were examined in the following species: American beech (*Fagus grandifolia*), black birch (*Betula lenta*), red maple (*A. rubrum*), and red oak (*Quercus rubra*). Significant relationships existed between soil  $\delta^{15}\text{N}$ , foliar  $\delta^{15}\text{N}$ , and foliar N content versus  $\text{NO}_2$  concentration in red maple seedlings across sites. Soil  $\delta^{15}\text{N}$  was the dominant driver of foliar  $\delta^{15}\text{N}$  patterns in red maple seedlings, regardless of mycorrhizal treatment. In adult tree species, there were significant correlations between foliar  $\delta^{15}\text{N}$  and both soil  $\delta^{15}\text{N}$  and  $\text{NO}_2$  concentration across sites. Using a multiple regression model,  $\text{NO}_2$  concentration explained 0%, 69%, 23%, and 45% of the residual variation in foliar  $\delta^{15}\text{N}$  remaining in American beech, red maple, red oak, and black birch, respectively, after accounting for soil  $\delta^{15}\text{N}$ . This chapter supports the use of natural abundance foliar  $\delta^{15}\text{N}$  to characterize the influence of soil  $\delta^{15}\text{N}$ , mycorrhizal fractionation, and foliar N uptake on plant N status in temperate forests.

Overall, these studies demonstrate the importance of the foliar N uptake pathway on plant metabolic processes and represent the first quantification of the magnitude of direct foliar uptake of gaseous NO<sub>2</sub> pollution in vegetation. Furthermore, this work indicates that if plants are able to assimilate significant quantities of gaseous N pollutants and incorporate them into organic compounds, direct foliar uptake of atmospheric N pollution may play a crucial role in the global N and carbon cycles through the alteration of photosynthesis and the removal of N pollutants from the atmosphere.

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CHAPTER 1:  
FOLIAR  $\delta^{15}\text{N}$  VALUES AS INDICATORS OF FOLIAR UPTAKE OF  
ATMOSPHERIC NITROGEN POLLUTION\*

**Abstract**

Vegetation is a significant sink for atmospheric nitrogen (N) pollutants in terrestrial ecosystems and, in N-limited systems, the foliar uptake of reactive N may represent a considerable source of N to plants. However, little research has examined the variation in foliar N assimilation among species at realistic concentrations or how this assimilation may influence foliar  $\delta^{15}\text{N}$  values. An understanding of the magnitude of foliar N uptake and whether foliar  $\delta^{15}\text{N}$  values may be used to track this assimilation of anthropogenic N is imperative for understanding the future N cycle. In the following chapter, I discuss several of the more promising laboratory and field efforts using  $\delta^{15}\text{N}$  to quantify foliar N uptake. In particular, I have used combined hydroponics-fumigation systems to address several of the unresolved questions associated with the foliar uptake of reactive N. Using this system, I evaluated tomato (*Lycopersicon esculentum*) and tobacco (*Nicotiana tabacum*), which contrast in the reduction site for metabolism, under low or high (50 $\mu\text{M}$  and 500 $\mu\text{M}$ ) root  $\text{NO}_3^-$  supply and exposed shoot systems to filtered or  $^{15}\text{NO}_2$ -enriched air (0 ppb, 20 ppb,

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and 40 ppb) for four weeks. A key finding from this work is that both species are capable of incorporating up to 15% of their total assimilated N using the foliar uptake pathway, although no significant variation in foliar uptake capacity between species was observed at the whole-plant level. Results indicate that NO<sub>2</sub> pollution may be used as a nutritional source under N-limiting conditions and may be traced through foliar  $\delta^{15}\text{N}$  values.

## **Introduction**

Among the cycles of elements important to life, the nitrogen (N) cycle has been the most perturbed beyond its natural state by human activities (Hopkins *et al.* 1998, Vitousek 1994, Vitousek *et al.* 1997). One manifestation of this perturbation is a dramatic increase in the amount of reactive N in the atmosphere. Anthropogenic emissions of atmospheric reactive N now exceed natural emissions and are the predominant source of atmospheric reactive N deposition to terrestrial ecosystems. Asia, Europe, and North America account for the majority of the current anthropogenic emissions and the highest levels of deposition. However, substantial increases in atmospheric reactive N are projected over the next 50 years for several other regions of the world as industrialization increases (Galloway *et al.* 2004, Phoenix *et al.* 2006). It is crucial that we improve our understanding of the potential sinks for reactive N as global inputs continue to increase.

Vegetation appears to be a significant sink for atmospheric reactive N and the direct foliar uptake of atmospheric N pollution may represent a considerable

source of N to plants (Hanson & Lindberg 1991, Hill 1971, Rondon & Granat 1994, Rondon *et al.* 1993). Plants are capable of assimilating the N in gaseous N compounds, including the reactive N oxides (“NO<sub>y</sub>”, including NO, NO<sub>2</sub>, HNO<sub>3</sub> and organic nitrates) and ammonia gases (“NH<sub>x</sub>”, including NH<sub>3</sub> and particulate NH<sub>4</sub><sup>+</sup>), and incorporating this N into organic compounds for growth (Gessler *et al.* 2002, Morikawa *et al.* 1998). The capacity for foliar uptake of reactive N is likely influenced by plant species characteristics and reactive N concentrations in the atmosphere (Morikawa *et al.* 1998). In this chapter, we review the potential of N stable isotopes, specifically foliar δ<sup>15</sup>N values, as an innovative and important tool for determining the magnitude and ultimate fate of atmospheric reactive N deposition to foliage globally.

Reactive N generation and redistribution globally is altering ecosystem function and the composition of natural plant assemblages both through growth-stimulating and phytotoxic effects (Bobbink & Roelofs 1995, Lea 1998, Pearson & Soares 1995, Vitousek *et al.* 1997, Wellburn 1990). Increasing the total reactive N input to an ecosystem can lead to several negative effects on ecosystem function such as soil and water acidification, increased loss of nutrients from the soil, changes to competitive interactions among plants, reductions in biodiversity, increased vulnerability of invasion, and nutrient imbalances in vegetation (Asner *et al.* 1997, Bobbink *et al.* 1998, Gilliam 2006). Moreover, plant performance can also be deleteriously affected through reductions in net photosynthesis, respiration, stomatal conductance, enzyme activities, and growth (Takahashi *et al.* 2005). Positive effects of increased reactive N deposition may include fertilization of N-limited ecosystems promoting increased carbon sequestration and plant growth (Beedlow *et al.*

2004). Although numerous studies have shown the impacts of total N deposition on ecosystem processes (Aber *et al.* 2003, Fahey *et al.* 1999, Galloway *et al.* 2003, Lovett 1994, Wesely & Hicks 2000), few have investigated the magnitude or influence of atmospheric N pollution assimilated directly by foliage (Sparks *et al.* 2001).

The ability of vegetation to incorporate N into plant metabolism from both leaves and roots suggests the fate of N deposited to an ecosystem may be fundamentally different depending upon the entry pathway. For example, the chemical products of foliar N assimilation (most commonly  $\text{NO}_3^-$ ) are plant nutrients and are directly incorporated from the leaf apoplast and used for growth (Gessler *et al.* 2000, Morikawa *et al.* 1998). In contrast, N additions to the soil surface during deposition may be incorporated into microbial biomass, volatilized, or leached. Furthermore, additional N to the soil surface may have other effects (e.g., changes in soil fertility, soil acidification, stripping of base cations, etc.). Thus, the direct entry of N through leaves is intriguing in that it could fundamentally change plant productivity through a direct influence on photosynthesis without the concomitant changes in soil chemistry often observed under high levels of soil N additions. However, it is still unclear if, and how, plant performance differs under N deposition to the soil (i.e., fundamentally altering the reactive N supply to root systems) versus changes in N additions from the atmosphere (i.e., changes in the magnitude of foliar uptake). Both forms of N entry into an ecosystem will likely increase together under most scenarios of intensified industrialization. However, various forms of gaseous reactive N have significantly different atmospheric lifetimes, suggesting plant communities arrayed across the landscape experience not

only unequal total N deposition, but different proportions of N through the root and foliar pathways.

There is some evidence to suggest the proportion of N incorporated through the foliar versus soil pathway may vary by environment. For instance, atmospheric reactive N is dominated by relatively reduced forms such as nitrogen dioxide ( $\text{NO}_2$ ) in industrialized areas. These compounds deposit more readily through the leaf stomata than to other surfaces in the environment (Sparks *et al.* 2001). At distances further from sources of N pollution, much of the reactive N is further oxidized to organic forms such as peroxyacetyl and isoprene nitrate. These compounds also readily enter plant stomata (Sparks *et al.* 2003) at a rate higher than deposition to other surfaces. In regions still further from sources of reactive N, the air mass is often dominated by nitric acid ( $\text{HNO}_3$ ). Nitric acid is very soluble in water and tends to deposit readily to many surfaces in the environment at a rate much faster than entry through the stomata (Byterowicz *et al.* 1998). Since the composition of atmospheric reactive N gases can vary across the landscape and are more or less likely to enter plant stomata, terrestrial ecosystems may receive differential amounts of N through the foliar uptake pathway.

Nitrogen stable isotope ratios ( $^{15}\text{N}/^{14}\text{N}$ ) are a useful tool for estimating the amount of anthropogenic N in the system and quantifying the uptake pathway into the plant [e.g., through soil  $\text{NO}_3^-$  or  $\text{NH}_4^+$ , dissolved organic N, foliar uptake via stomata, cuticular diffusion, etc. (Robinson *et al.* 1998, Robinson *et al.* 2000)] because forms of N available to plants often have varying isotopic compositions (Evans 2001, Kolb & Evans 2002). This may be especially

relevant to industrialized, urban regions where pollutant compounds contribute a significant amount of N input via dry and wet deposition to ecosystems. Consequently, the difference in source isotopic composition can provide a natural tracer for the assessment of foliar uptake and assimilation of reactive N pollution in terrestrial ecosystems (Handley & Raven 1992, Handley & Scrimgeour 1997). Specific to foliar uptake, if the foliar incorporated source is significantly different from the soil source, then the foliar  $\delta^{15}\text{N}$  value will be representative of both sources and can be used to quantify the proportion of each N source incorporated into plant biomass. If plants are able to assimilate significant quantities of gaseous N pollutants and incorporate them into organic compounds, foliar  $\delta^{15}\text{N}$  will be an important tool to examine the role foliar uptake of atmospheric N pollution plays in the global N and carbon cycles through the alteration of photosynthesis and the removal of N pollutants from the atmosphere.

In this chapter, I discuss the use of foliar  $\delta^{15}\text{N}$  values as a tool for determining the magnitude and impact of gaseous reactive N directly entering the foliage of vegetation. Although not an exhaustive treatment, I place this discussion within the context of the causative factors defining foliar  $\delta^{15}\text{N}$  values. I also describe the current understanding of foliar  $\delta^{15}\text{N}$  values to provide a background upon which foliar uptake of reactive N can be considered.

### **Nitrogen sources to terrestrial vascular plants**

The N sources assimilated by plants often have different isotopic compositions and many studies have used foliar  $\delta^{15}\text{N}$  values to indicate the  $\delta^{15}\text{N}$  of the

dominant N source used by a plant and as a measure of N sources from both soil and atmospheric deposition (Evans 2001). However, we now know that foliar  $\delta^{15}\text{N}$  values represent the integration of a range of processes (Evans 2001, reviewed by Handley & Scrimgeour 1997, Robinson 2001, Stewart *et al.* 2002) and can vary by as much as  $\pm 10\text{‰}$  in co-existing species (Handley & Scrimgeour 1997). The presence of multiple N sources with distinct isotopic values, differential processing of soil N pools (e.g. temporal and spatial patterns of net mineralization and nitrification), temporal and spatial variation in N availability, mycorrhizal interactions, and alterations in plant N demand can all influence foliar  $\delta^{15}\text{N}$  (Figure 1.1). Identifying and differentiating the sources of N available to plants is necessary for a variety of ecological questions including accurately interpreting foliar  $\delta^{15}\text{N}$  values. Further, additional understanding of how specific N species deposited to terrestrial ecosystems impact foliar  $\delta^{15}\text{N}$  values will provide us a tool to link plant physiological responses, terrestrial ecosystem function, and atmospheric chemistry. In this section, we briefly discuss various sources of plant-available N and their potential influences on foliar  $\delta^{15}\text{N}$ .

### *Soil nitrogen sources*

Plants derive N primarily from the soil in the form of ammonium ( $\text{NH}_4^+$ ), nitrate ( $\text{NO}_3^-$ ), and, secondarily, from organic N. Plant available soil N originates from decomposition of organic matter, biological N fixation, additions of N in organic or inorganic fertilizers, and deposition originating from fossil fuel combustion and animal production (Hopkins *et al.* 1998). There is enormous variability in the isotopic composition of the soil N pool available to plants. For example,



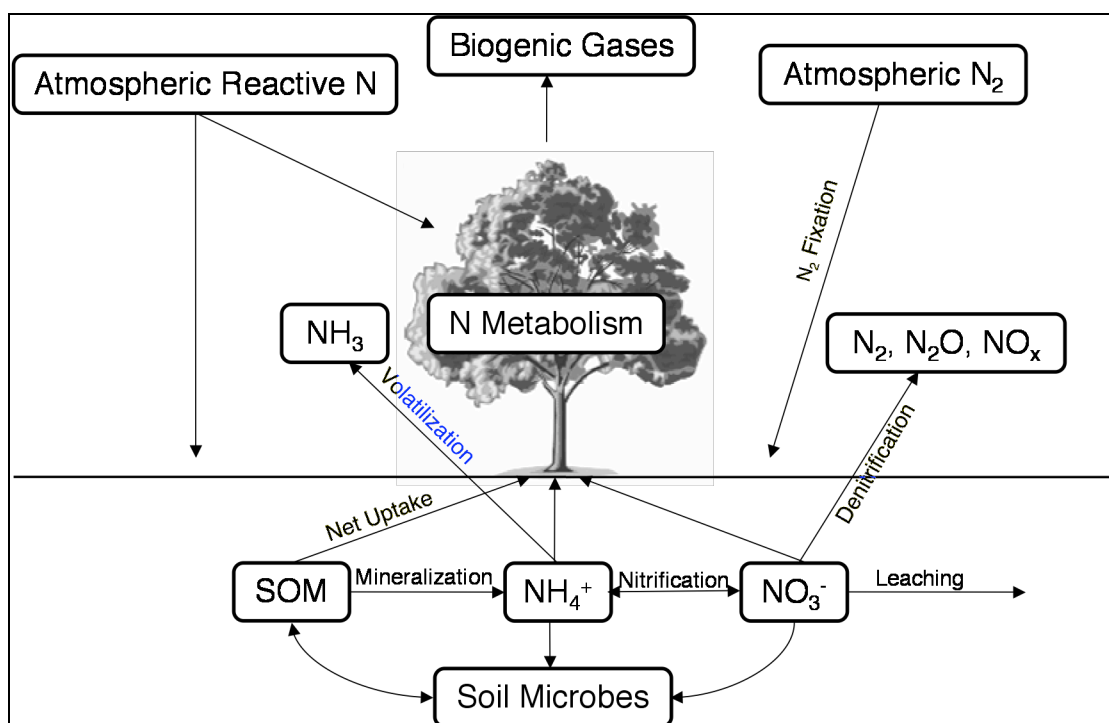


Figure 1.1. Primary source pools and uptake pathways in the N cycle. Boxes represent pools and arrows represent fluxes. Various N transformations in the soil and atmosphere lead to varying amounts of  $^{15}\text{N}$  enrichment or depletion in natural ecosystems, which can vary greatly with geographical location and environmental conditions. SOM represents soil organic matter derived from plant, microbial, and faunal organic tissues (adapted from Dawson *et al.* 2002).

evidence suggests that bulk soil  $\delta^{15}\text{N}$  and the  $\delta^{15}\text{N}$  of soil  $\text{NO}_3^-$  and  $\text{NH}_4^+$  diverge from each other at various timescales. This suggests that foliar  $\delta^{15}\text{N}$  may be interpreted as the integrated signal of  $\delta^{15}\text{N}$  of all plant-available N forms over the growth history of a particular leaf. However, the leaf signal may not represent the current status of soil  $\delta^{15}\text{N}$  at the time of sampling. Symbiotic interactions with fungi and  $\text{N}_2$ -fixing bacteria may also obscure the interpretation of the foliar  $\delta^{15}\text{N}$  value (Evans 2001). Further, observations have suggested that much of the variation in foliar  $\delta^{15}\text{N}$  among co-existing species may be due to the internal metabolism of fungi participating in the mycorrhizal

association (Handley *et al.* 1998). For example, under N-limited conditions, plants interacting with mycorrhizae will have a lower  $\delta^{15}\text{N}$  compared to the fungus due to fractionation during N transfer from the symbiont to the host plant (Evans 2001).

Differential uptake of multiple N sources with different  $\delta^{15}\text{N}$  values, differences in fractionation during uptake and assimilation, and physiological differences between plants and mycorrhizal symbionts are significant obstacles to the use of foliar  $\delta^{15}\text{N}$  values as a tool to assess the foliar uptake of reactive N.

Currently, there is no adequate method for isolating and analyzing  $\delta^{15}\text{N}$  in soil N pools that are available for plant uptake. Consequently, the influence of foliar uptake on foliar  $\delta^{15}\text{N}$  is confounded by variations in the soil system.

Variability in substrate supply, abiotic conditions, organism assemblages and their demand for N influence foliar  $\delta^{15}\text{N}$  values to a degree beyond that of pollution additions to the soil surface or foliage, making most field based studies challenging. Ultimately, enhancing our understanding of the fractionations and transformations occurring in and between soil N pools and whether there are systematic correlations among pools will dramatically improve our ability to use foliar  $\delta^{15}\text{N}$  as an indicator and allow us to examine foliar N uptake in natural systems.

#### *Atmospheric nitrogen sources*

Atmospheric N deposition can affect plant functioning via both foliar and soil uptake, with different effects on plant growth depending on the mode of entry and the type of N compound and plant species (Stulen *et al.* 1998).

Atmospheric reactive N can enter terrestrial ecosystems via wet, dry, and occult deposition. Most forms of atmospheric reactive N are generated from the partial oxidation of  $N_2$  at high temperatures and via the volatilization of animal waste. Total oxidized reactive N ( $NO_y$ ) and ammonia gases ( $NH_x$ ) are the most prevalent atmospheric reactive N sources, can be transported long distances, and are eventually deposited on terrestrial ecosystems as  $NO_3^-$  and  $NH_4^+$  in precipitation, as gases, or as particles. Major sources of  $NO_y$  typically include power production, transportation sources, off-road mobile sources, and other industrial sources (EPA 1998). Sources of  $NH_x$  include both natural plant and soil emissions and anthropogenic emissions from agricultural and animal waste products (Srivastava 1992). In addition to inorganic N sources, there are several sources of naturally-occurring organic N, including plant pollen and sea-spray droplets (Driscoll *et al.* 2003). Atmospheric organic N may also be derived from anthropogenic inorganic N compounds reacting with hydrocarbons or organic particles in the atmosphere (Prospero *et al.* 1996) and generally comprises 30% of total atmospheric reactive N deposition (Neff *et al.* 2002). All of these compounds have distinct deposition velocities, travel different distances from point sources of N pollution, and have different probabilities of being deposited to the soil or directly to vegetation. For example, recent work by Sparks *et al.* (2003) and Turnipseed *et al.* (2005) has shown that peroxyacetylnitrate (PAN), an organic N pollutant and the dominant form of reactive N transported long distances in the atmosphere, is readily taken up through plant foliage, primarily deposits directly to vegetation via foliar uptake and is less likely to be deposited to the soil N pool, making it a potential source of N to the plant that predominantly enters through the foliar uptake pathway.

### *Wet deposition*

Most forms of reactive gas-phase N will eventually be oxidized to a form [most commonly nitric acid ( $\text{HNO}_3$ )] that is highly soluble and easily scavenged by particles or water droplets. These compounds are deposited to ecosystems within precipitation, low clouds and fog. This process is collectively termed wet deposition and refers generally to the transfer of soluble compounds from the atmosphere to the earth's surface. Wet reactive N deposition contains a variety of N compounds, most of which are available for biological utilization, including inorganic ( $\text{NO}_3^-$ ,  $\text{NO}_2$ ,  $\text{NH}_4^+$ ) and organic (amino acids, PAN, urea) chemical species (Peierls & Paerl 1997). Cloud deposition, which occurs through impaction of fog droplets on exposed surfaces, can contribute between 25% and 50% of total N deposition in high-elevation areas of the Northeastern United States (Anderson *et al.* 1999). These compounds can be deposited directly onto leaf surfaces and later incorporated into the plant tissue or deposited to the soil surface and added to the soil pool.

### *Dry deposition*

In the absence of an aqueous phase, gases and dry particles can be transported to and deposited into terrestrial ecosystems in the form of dry deposition. Dry deposition of gaseous N pollutants has been shown to be of a similar magnitude (or potentially greater) to wet deposition in many regions (Holland *et al.* 2004). Monitoring efforts in North America and Western Europe report that reactive N deposition flux to terrestrial ecosystems via dry deposition is dominated by  $\text{NO}_2$ ,  $\text{NH}_3$ ,  $\text{HNO}_3$  and a relatively small amount of

particle-nitrate. However, most monitoring efforts only assess inorganic forms of N and Day et al. (2003) suggest that the dry deposition of organic N could be comparable to that of  $\text{HNO}_3$ . Dry deposition estimates are still highly uncertain in most areas, and methodology is still an active area of research. It is desirable for ecologists to not only evaluate the magnitude of gaseous N deposition on terrestrial ecosystems, but to also monitor the isotopic composition of the deposited N and the resulting impact on foliar  $\delta^{15}\text{N}$  values.

### **Foliar assimilation of atmospheric nitrogen pollutants**

The impact of atmospheric reactive N deposition to leaves depends on the physical and chemical characteristics of the pollutant and the plant species, including adsorption of the compound to the leaf surface, compound solubility within the internal plant cell wall and its reactivity with cellular components, and physiological factors such as stomatal conductance and the rate of N metabolism. Foliar uptake occurs through two routes: stomatal diffusion and uptake via the cuticle, although the latter is negligible for most chemical species. Cuticular uptake of N compounds (most commonly  $\text{HNO}_3$ ) refers to the active or passive transport of a molecule through the lipid bilayer of the plasmalemma into the cytosol. Gaseous N pollutants enter the leaf through one of these two routes and are assimilated by different biochemical pathways (Figure 1.2). For example, ammonia gas diffuses through the stomata and is dissolved in the apoplastic space to form  $\text{NH}_4^+$ . Ammonium is transported into the cytoplasm where it is either partitioned into storage or assimilated by the glutamine synthetase/glutamate synthase cycle (Stulen et al. 1998). In contrast, after uptake via diffusion through the stomata,  $\text{NO}_2$  enters the

substomatal cavity of the leaf and disproportionates to form  $\text{NO}_3^-$  and nitrite ( $\text{NO}_2^-$ ). Eventually,  $\text{NO}_2^-$  is reduced to  $\text{NH}_2$  via nitrate reductase in the cytosol and nitrite reductase in the chloroplast, used in amino acid synthesis, and incorporated into primary N metabolism (Weber *et al.* 1998, Wellburn 1994).

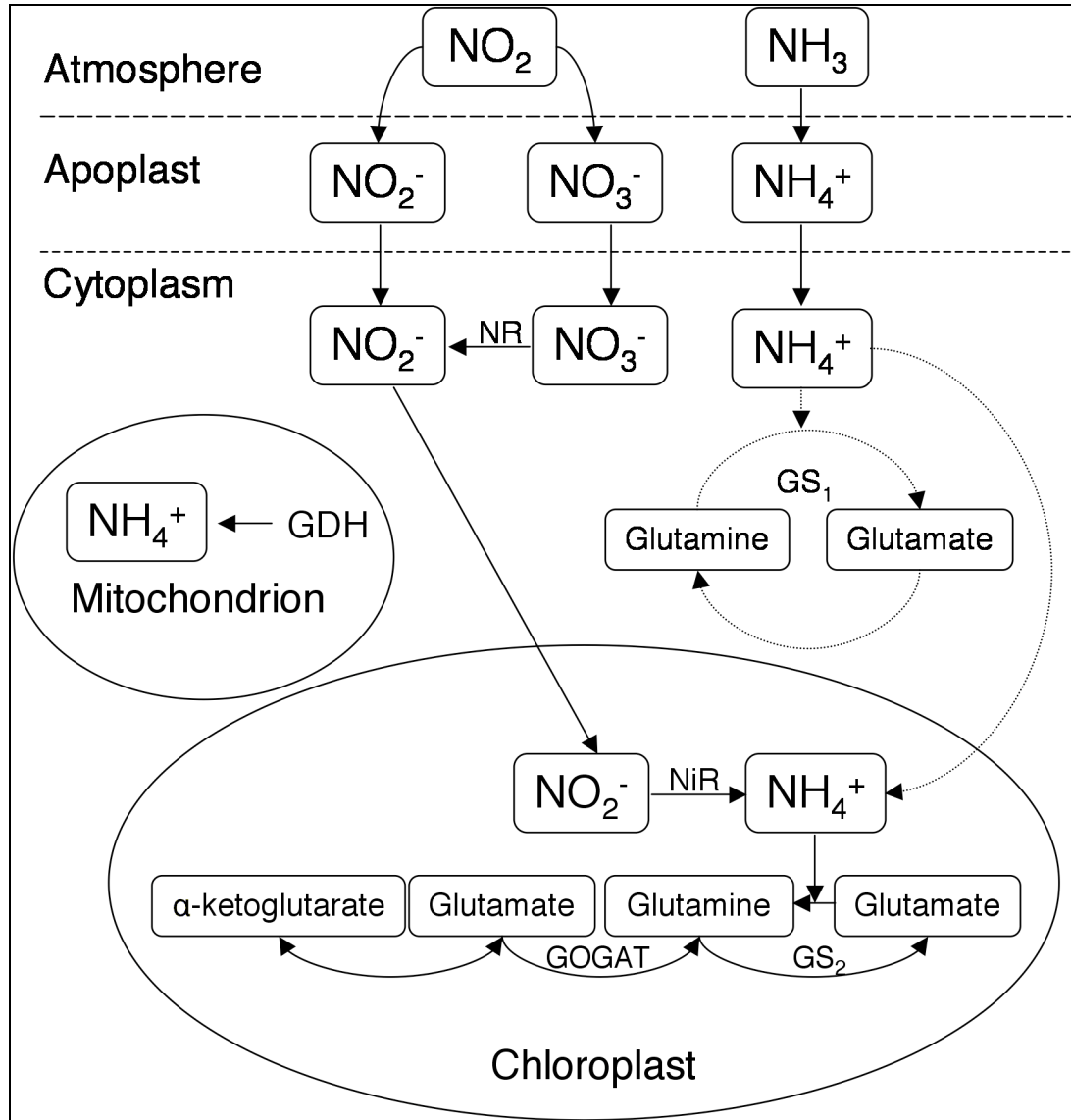


Figure 1.2. Primary metabolic processes involved in the foliar uptake and assimilation of atmospheric ammonia ( $\text{NH}_3$ ) and nitrogen dioxide ( $\text{NO}_2$ ).  $\text{NH}_3$  and  $\text{NO}_2$  enter the apoplast via stomatal or cuticular uptake, are assimilated into primary N metabolism by various biochemical pathways. Dashed lines indicate the possible role of cytosolic **GS<sub>1</sub>** or chloroplastic **GS<sub>2</sub>** in  $\text{NH}_4^+$  assimilation derived from atmospheric  $\text{NH}_3$  (adapted from Stulen *et al.* 1998).

## **Contribution of foliar uptake to total plant nitrogen assimilation**

Vegetation has the capacity to assimilate atmospheric sources of N both through root and foliar pathways. Through modeling, direct foliar uptake of reactive N has been estimated to contribute 3 - 16% of the plant N demand for new growth tissue (Holland *et al.* 2004). Although this is a small proportion of the total N required for growth, N directly assimilated via foliar uptake is a direct addition to plant metabolism, would not necessarily influence soil acidity, and would likely influence plant productivity and carbon assimilation in N-limited terrestrial ecosystems. This additional source of N to plants could also increase plant growth under increased atmospheric carbon dioxide (CO<sub>2</sub>). Carbon dioxide fertilization effects, when they are observed, are often not sustained as the plant community becomes limited by some secondary limitation. This limitation is often hypothesized to be N availability in many ecosystems (Beedlow *et al.* 2004). Plants become N deficient because of the relatively higher availability of carbon substrate for photosynthesis. Under such a scenario, N available through foliar uptake would enhance or at least sustain more rapid growth under elevated CO<sub>2</sub>. Experiments directly quantifying the proportion of N assimilated through foliage or demonstrating a positive relationship between foliar uptake of N and elevated CO<sub>2</sub> have not yet been forthcoming.

### *Variation in foliar nitrogen assimilation via the foliar uptake pathway*

The ability of vegetation to incorporate atmospheric reactive N via foliar uptake has been demonstrated, but few studies have examined the variation in leaf N

assimilation among plant species. For example, Morikawa *et al.* (1998) documented a 600-fold variation in leaf N assimilation among 217 plant taxa. However, most studies, including this one, have been limited by the use of unnaturally high fumigation levels and limited mechanistic explanations as to why some species have a higher capacity for foliar uptake than others.

Reactive N uptake by leaves is strongly controlled by diffusion processes (Thoene *et al.* 1991, Weber & Rennenberg 1996) and variation in stomatal dynamics among species likely explains some of the observed variation in foliar uptake capacity. However, variation in foliar uptake of reactive N among species is likely controlled by factors in addition to diffusional resistance. For example, if stomatal conductance remains fairly constant, variations in the biochemical capacity for NO<sub>2</sub> assimilation would alter the sub-stomatal air cavity NO<sub>2</sub> concentration, and subsequently influence the NO<sub>2</sub> concentration gradient into the leaf and the rate of foliar NO<sub>2</sub> uptake (Hereid & Monson 2001). This 'internal' resistance to uptake is often termed the mesophyll resistance and has been suggested to be controlled by a number of factors including: (1) the permeability of NO<sub>3</sub><sup>-</sup>/NO<sub>2</sub><sup>-</sup> ions through cell membranes and walls (Ammann *et al.* 1995), (2) the site of the activity in the primary nitrate assimilation pathway through which NO<sub>y</sub> is metabolized (Wellburn 1990), and (3) the activity of ascorbic acid and other antioxidant compounds in the leaf apoplast (Teklemariam & Sparks 2004).

Recent work by Eller and Sparks (2006) has simultaneously investigated the hypothesized controlling factors over reactive N flux into leaves. In this study, the investigators measured stomatal conductance as an estimate of the



diffusional resistance to gases entering the leaf, the concentration of apoplastic ascorbate to examine the reaction rates between gases and chemicals within cell walls of leaf tissue, and the activity of the enzyme nitrate reductase (NR) as a proxy for the transport rate of  $\text{NO}_3^-$  out of the apoplast and then incorporated the strengths of each of these controlling factors into a multiple regression model describing leaf uptake of  $\text{NO}_2$ . As in previous studies, stomatal conductance was found to be the primary control over the foliar uptake of  $\text{NO}_2$ . However, NR activity and leaf ascorbate concentrations explained significant variance in  $\text{NO}_2$  leaf flux, suggesting that enzyme activities and antioxidant concentrations in the cell wall likely underlie the differences we observe across species in mesophyll resistance to  $\text{NO}_2$  uptake. For example, NR activity is variable across species and such differences could account for the broad variation in rates of  $\text{NO}_2$  uptake (Morikawa *et al.* 1998) because plants expressing higher NR activities may have higher  $\text{NO}_2$  uptake capacity at elevated  $\text{NO}_2$  levels where the cell wall may be saturated with  $\text{NO}_3^-/\text{NO}_2^-$  (Eller & Sparks 2006). Furthermore, antioxidants such as ascorbate could also contribute to uptake capacity depending on their concentration and associated ability to react with oxidants such as  $\text{NO}_2$ . Ultimately, the concentration of ascorbate may control the steady-state flux of  $\text{NO}_2$  into a leaf. Increasing our knowledge of these leaf biochemical processes will aid in our understanding of the existing variation in foliar uptake capacity among species.

### **Foliar $\delta^{15}\text{N}$ as a tool to determine the magnitude of foliar nitrogen uptake**

The differences in  $\delta^{15}\text{N}$  between atmospheric N, plant-available soil N, and N

derived from biological fixation may be used as a natural tracer in plant systems. Further, the  $\delta^{15}\text{N}$  of atmospheric N compounds produced from anthropogenic activities may be significantly different from the natural, background N sources in the soil (Freyer 1991). This anthropogenic signal in foliar  $\delta^{15}\text{N}$  has been reported in several studies (Ammann *et al.* 1999, Qiao & Murray 1997, Siegwolf *et al.* 2001) and may be a reliable indicator of N pollution addition. For example, the  $\delta^{15}\text{N}$  of atmospheric  $\text{NO}_y$  pollution from traffic exhausts and industrial combustion processes is mostly positive (-1‰ to +5‰) and atmospheric  $\text{NH}_3$  originating from the volatilization of animal waste is usually very depleted (Heaton 1986, Macko & Ostrom 1994) compared to natural sources of inorganic N (e.g.,  $\text{NO}_3^-$  from mineralization) that are usually negative [between -5‰ and -2‰ (Nadelhoffer & Fry 1994)]. Because of these differences, the relative magnitude of different N sources can be observed at the leaf level (Ammann *et al.* 1999, Siegwolf *et al.* 2001). Only if there are differences in  $\delta^{15}\text{N}$  among potential N sources can  $\delta^{15}\text{N}$  be used as an indicator for any of these sources (Robinson 2001).

Assuming the  $\delta^{15}\text{N}$  of the various sources are known, the amount of N assimilated by the plant can be quantified. The final isotopic composition of the leaf tissue is a combination of the various source values and masses. Take, for example, a plant that receives N from two sources: root uptake of soil  $\text{NO}_3^-$  and foliar uptake of atmospheric  $\text{NO}_2$ . If the isotopic ratios of both sources are known, we can use a two ended mixing model and the proportion of N obtained from foliar uptake ( $X_{\text{foliar}}$ ) is:

$$X_{\text{foliar}} = \frac{\delta^{15}\text{N}_{\text{plant}} - \delta^{15}\text{N}_{\text{soil}}}{\delta^{15}\text{N}_{\text{atmosphere}} - \delta^{15}\text{N}_{\text{soil}}} \quad [1]$$

where  $\delta^{15}\text{N}_{\text{plant}}$ ,  $\delta^{15}\text{N}_{\text{soil}}$ , and  $\delta^{15}\text{N}_{\text{atmosphere}}$  are the N isotope compositions of the leaf, soil, and atmospheric N sources, respectively.

### **Current challenges for using foliar $\delta^{15}\text{N}$ as a tool to determine the magnitude of foliar nitrogen uptake**

The obstacles for using mixing models like the one described above and interpreting the foliar  $\delta^{15}\text{N}$  value lie in the presence of simultaneously available multiple N sources and currently unknown fractionation events that occur during N transformation, uptake, and assimilation. As discussed in section 2.2, this is a significant issue in the soil system allowing the generation of various foliar  $\delta^{15}\text{N}$  values driven by differences in soil N cycling. Although the mechanisms are largely unknown, differences between the  $\delta^{15}\text{N}$  of plant material vs. soil  $\delta^{15}\text{N}$  suggest some within-plant fractionation of N (Comstock 2001, Robinson *et al.* 1998). In addition, foliar uptake of reactive N presumably exhibits an array of largely unknown fractionation events as well. For example, N gases assimilated through the stomata both have variable values in the atmosphere and are likely influenced by the same fractionation events caused by diffusion that influence the incorporation of  $\text{CO}_2$  during photosynthesis. Therefore, a large fractionation is expected to occur during the diffusion of  $\text{NO}_2$  into the leaf. Fractionations associated with transport into plant cells and N reduction enzymes, although not yet quantified, are also likely to occur. Further fractionation likely occurs during diffusion and biochemical assimilation pathways by a fine-scale separation of pools. As an example, in the substomatal cavity,  $\text{NO}_2$  rapidly disproportionates in water within the cell wall and is converted to  $\text{NO}_3^-$ . Accurately defining the difference in isotopic

composition between the gaseous  $\text{NO}_2$  in the air and  $\text{NO}_3^-$  in the cell wall would describe the fractionation due to stomatal diffusion and disproportionation of  $\text{NO}_2$ . However, for foliar uptake of reactive N, none of these fractionation events have been adequately resolved.

Developing a more complete understanding of the source values and fractionation events associated with foliar  $\delta^{15}\text{N}$  in the plant-soil system is necessary to predict the importance of various N sources on the plant N budget (Handley *et al.* 1998). Promising techniques are currently being developed and used to elucidate some soil processes, such as dual-isotope techniques [e.g.,  $\delta^{15}\text{N}$  and  $\delta^{18}\text{O}$  in  $\text{NO}_3^-$  (Durka *et al.* 1994)]. Ultimately, a solid understanding of these processes will be necessary for the production of a mechanistic model describing how  $\delta^{15}\text{N}$  changes during plant N acquisition and assimilation from both foliar and soil uptake pathways (Dawson *et al.* 2002).

### **Promising methods using foliar $\delta^{15}\text{N}$ as an indicator of direct leaf nitrogen assimilation**

A possible strategy for quantifying the foliar uptake of atmospheric reactive N pollution using foliar  $\delta^{15}\text{N}$  values is to enhance the magnitude of differences in  $\delta^{15}\text{N}$  in the various N pools against a background level such that the difference in signal may provide a quantifiable tracer. In the laboratory, the external chemical and isotopic composition of the N source(s) can be identified and controlled. We describe this type of laboratory experiment in the following section. In the field, a significant  $^{15}\text{N}$ -enrichment of the gas-phase N source would be necessary to create a large enough signal separation. The naturally

occurring range of  $\delta^{15}\text{N}$  found in nature is generally no more than  $\pm 20\%$  (Handley & Scrimgeour 1997). Therefore, the  $^{15}\text{N}$ -enrichment of the gas source would need to be large enough to overcome this natural variation. Systems enriched in  $^{15}\text{N}$  ameliorate many of the problems of interpretation brought about by fractionation among N pools and are useful for following the movement of N through a plant and for determining rates of N assimilation within the plant (Dawson *et al.* 2002, Nadelhoffer & Fry 1994). In the following sections, we discuss several of the more promising laboratory and field efforts using  $^{15}\text{N}$  to quantify foliar N uptake.

### *Hydroponics*

Hydroponics systems allow for the control of the isotopic composition of the nutrient solution N and, if adequately replenished, remain constant through time. Traditional hydroponics systems have been used to quantify foliar  $\delta^{15}\text{N}$  values (Evans *et al.* 1996, Pritchard & Guy 2005, Qiao & Murray 1997, Qiao & Murray 1998, Yoneyama & Kaneko 1989) and to explore the impact of  $\text{NO}_2$  fumigation on root uptake of N (Muller *et al.* 1996). However, these studies did not directly address the magnitude nor the consequences of foliar uptake of reactive N.

We have used combined hydroponics-fumigation systems to address several of the unresolved questions associated with the foliar uptake of reactive N (Figure 1.3).

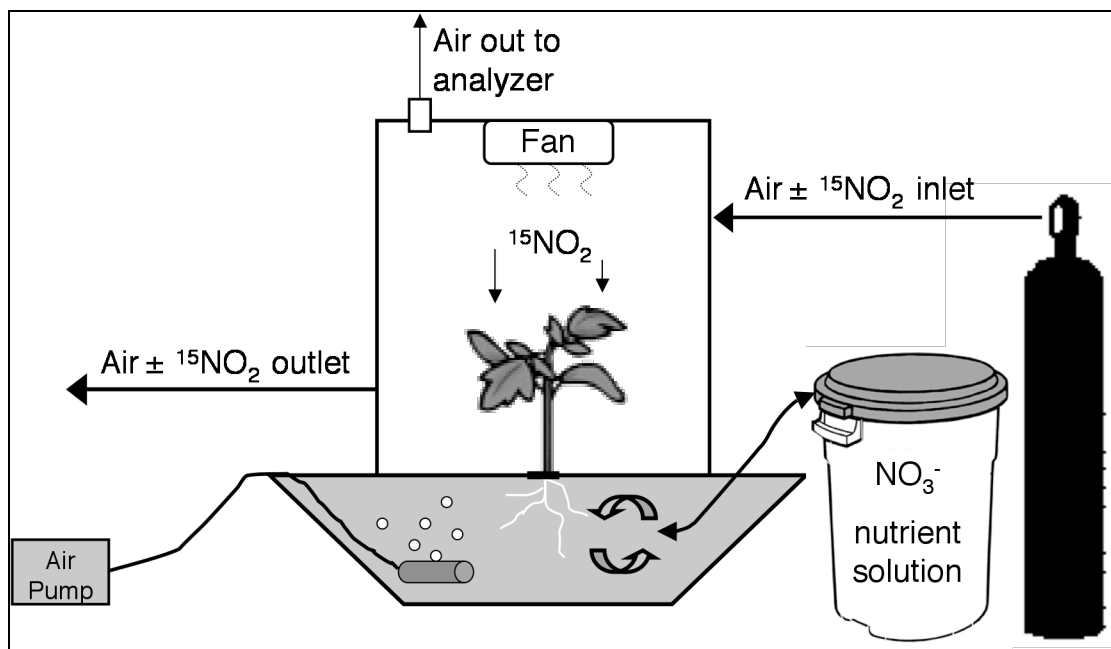


Figure 1.3. Design of an individual hydroponics fumigation system used with seedlings for long-term (weeks) experiments. The root system is suspended in the nutrient solution while the shoot system is contained within the fumigation chamber. The seedling is sealed at the root-shoot junction with modeling clay to isolate the N sources. The nutrient solution is refreshed on a weekly basis and aerated by an aquarium pump. The  $\text{NO}_2$  concentration of the incoming air to the fumigation chamber is held constant by mixing concentrated  $^{15}\text{NO}_2$  gas with clean air by using mass flow controllers. It is vigorously mixed in the fumigation chamber by a fan.

Plants are transplanted into the system with the root systems suspended in nutrient solution and the shoot systems enclosed in a fumigation chamber. Using this system, we can simultaneously and independently control the forms, external concentrations, and isotopic compositions of N sources available to plant root and shoot systems. After specific exposure protocols (i.e., different combinations of N gas concentrations to the leaves and N fertilizer to roots), plant biomass and isotopic composition measurements are used to quantify the proportion of each N source utilized and the overall influence of the exposure protocol on plant growth. Using this system, I have

determined the partitioning of plant N among sources and the potential magnitude of  $\text{NO}_2$  assimilation in tomato (*Lycopersicon esculentum*). Depending on the root availability of  $\text{NO}_3^-$ ,  $\text{NO}_2$  derived N in leaf tissue varied between 1 and 7% at the end of the fumigation (Figure 1.4).

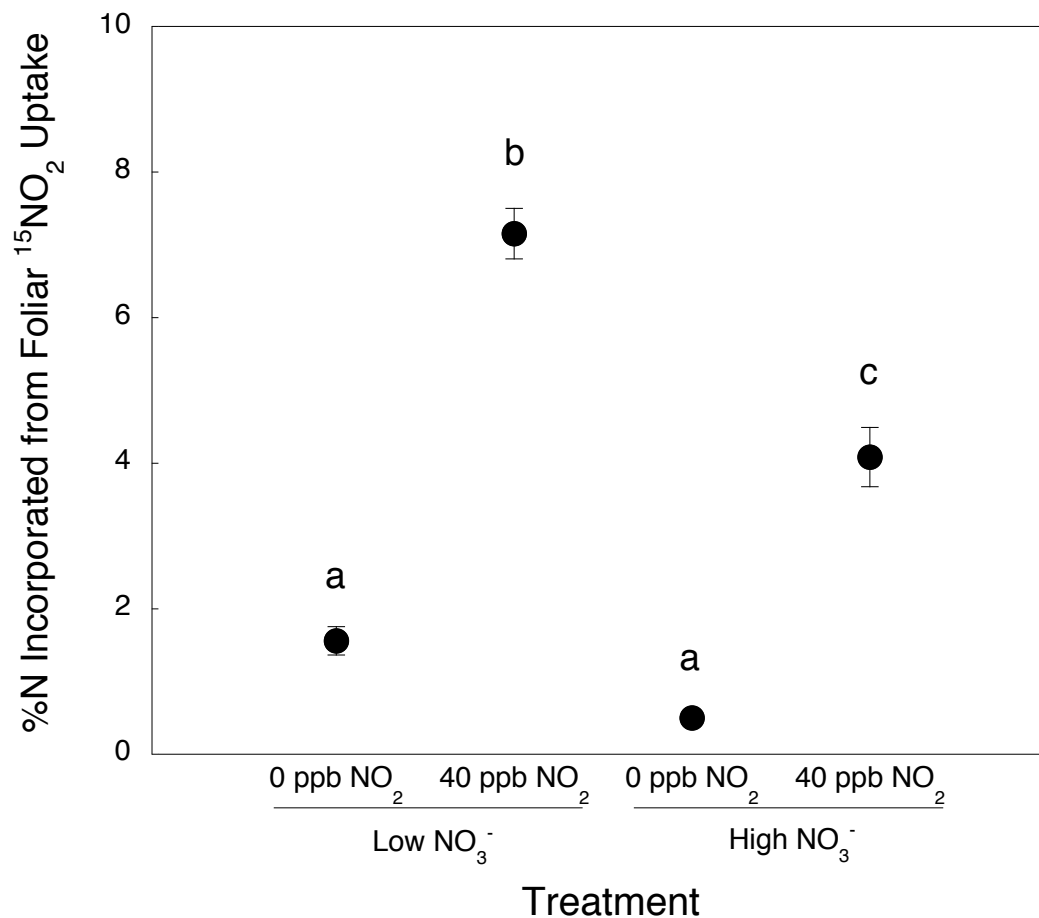


Figure 1.4. Proportion (%) of total N content attributable to the direct foliar uptake of  $^{15}\text{NO}_2\text{-N}$  in *L. esculentum*. Data are means  $\pm$  1 SE. Data with distinct lowercase letters indicate significant differences. From Vallano and Sparks (unpublished).

We have also begun to address the variation in foliar N assimilation among plant species at realistic concentrations and how this assimilation may influence foliar  $\delta^{15}\text{N}$  values. Using the same hydroponics-fumigation system

to quantify species differences in foliar NO<sub>2</sub> assimilation, we have examined differences in growth and foliar  $\delta^{15}\text{N}$  values in two species that contrast in their N reduction strategies: tomato (*Lycopersicon esculentum*) and tobacco (*Nicotiana tabacum*). In natural and cultivated systems, *L. esculentum* primarily reduces N in the roots, while *N. tabacum* primarily reduces N in the leaves, suggesting that *N. tabacum* may have an increased capacity to incorporate and assimilate atmospheric N sources via the leaves. Seed-grown plants were transplanted into the system and exposed to low or high (50  $\mu\text{M}$  and 500  $\mu\text{M}$ ) root NO<sub>3</sub><sup>-</sup> supply and filtered or <sup>15</sup>NO<sub>2</sub>-enriched air (0 ppb, 20 ppb, and 40 ppb) for four weeks. A key finding from this work is that both species are capable of incorporating up to 15% of their total assimilated N using the foliar uptake pathway, although no significant variation in foliar uptake capacity between species was observed at the whole-plant level (Figure 1.5).

This result suggests that the foliar uptake of NO<sub>2</sub> contributes a significant proportion of N to plant metabolism and is consistent with modeled values of foliar uptake in the literature (Ammann *et al.* 1999, Muller *et al.* 1996). We also observed a NO<sub>2</sub>-induced stimulation in whole-plant biomass production in both species similar to those observed in some fumigation studies (Muller *et al.* 1996, Siegwolf *et al.* 2001). These results suggest that NO<sub>2</sub> is potentially beneficial to biomass production and can change whole-plant allocation patterns under certain conditions. Further analysis of NR activity is necessary to highlight any species differences in foliar uptake capacity. Obviously, hydroponics-fumigation experiments like the ones we describe here are not an adequate simulation of natural conditions. However, they do lend significant insight into the transport of N pollutants into plants, and the physiological



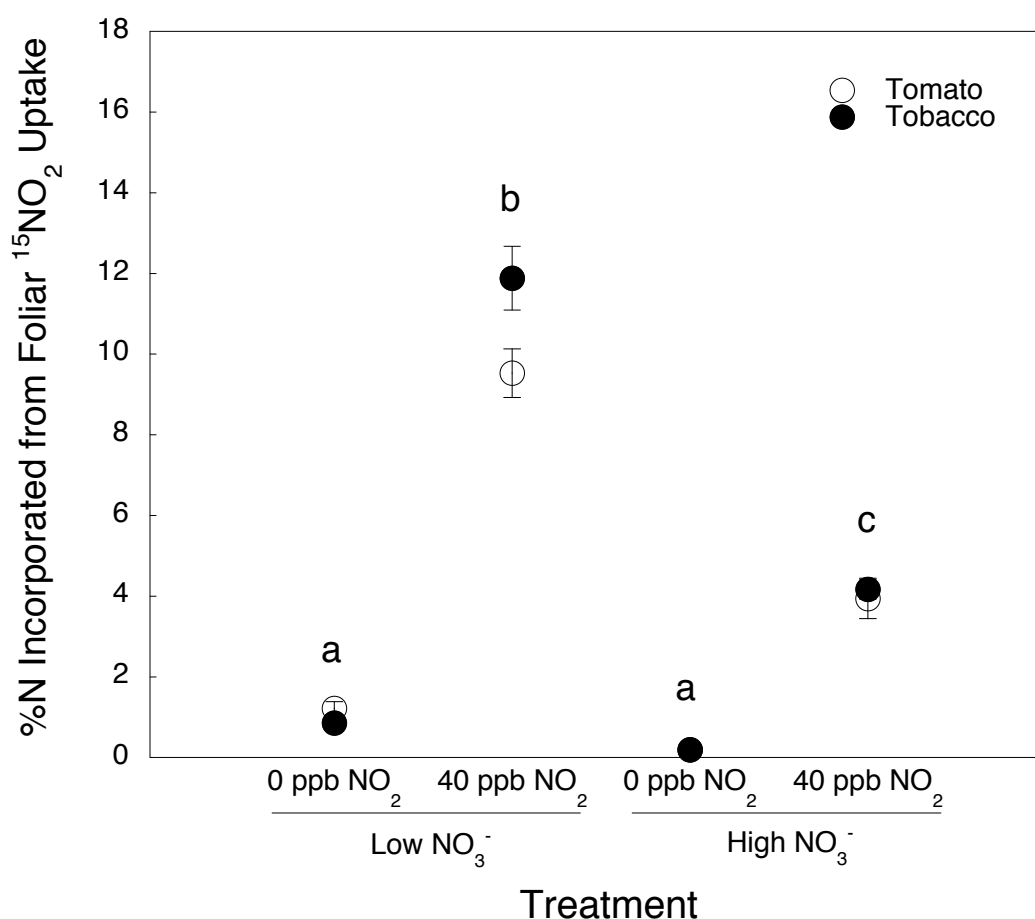


Figure 1.5. Proportion (%) of total N content attributable to the direct foliar uptake of  $^{15}\text{NO}_2\text{-N}$  in *N. tabacum* (●) and *L. esculentum* (○). Data are means  $\pm$  1 SE. Data with distinct lowercase letters indicate significant differences for both species. From Vallano and Sparks (unpublished).

mechanisms that influence the capacity of various plant species to incorporate atmospheric reactive N pollution via foliar uptake. Our future work using these systems will include multiple species comparisons of foliar uptake capacity, combined applications of multiple atmospheric N pollution sources, and quantification of the fractionation events associated with the foliar N uptake and how this influences foliar  $\delta^{15}\text{N}$  values.

### *Field studies*

Controlled laboratory experiments have yielded promising results for using foliar  $\delta^{15}\text{N}$  measurements as indicators of reactive N uptake through leaves. However, can we use these, or similar, methods to estimate the proportion of foliar uptake of anthropogenic reactive N in natural systems? Multiple field studies have used foliar  $\delta^{15}\text{N}$  values to estimate the contribution of total atmospheric N pollution in terrestrial ecosystems (Ammann *et al.* 1999, Gessler *et al.* 2002, Siegwolf *et al.* 2001, Wania *et al.* 2002). However, this work did not attempt to separate uptake pathways and most were likely complicated by many of the soil factors discussed earlier (e.g., mycorrhizal effects,  $\text{N}_2$  fixation, heterogeneity of soil N sources and transformations). Currently, no consistent mechanistic relationship has been reported between atmospheric N pollution, foliar uptake of atmospheric N pollution, and foliar  $\delta^{15}\text{N}$  values.

In temperate forest ecosystems, high concentrations of total N pollution addition to ecosystems have resulted in the  $^{15}\text{N}$  enrichment of soil and plants (Stewart *et al.* 2002). Measurements of foliar  $\delta^{15}\text{N}$  in pine needles representing both the soil and foliar uptake of N deposition in heavily polluted areas of Germany varied by an order of magnitude depending on the plants proximity to various emission sources (Jung *et al.* 1997). It has been suggested these trends are the result of the loss of  $^{15}\text{N}$  depleted N from soil and plants due to volatilization causing enrichment of the residual N fraction (Hogberg *et al.* 1999). This is likely true, but the role and importance of foliar uptake in determining the foliar  $\delta^{15}\text{N}$  value and what this means in terms of

plant function is still unknown and likely an important factor in these measurements.

Ammann et al. (1999) attempted to quantify the influences of soil and foliar uptake pathways on pollutant N assimilation by sampling whole plant communities over a growing season and examining the resulting patterns of foliar  $\delta^{15}\text{N}$ . They estimated  $\text{NO}_2$  uptake in *Picea abies* by analyzing atmospheric  $\text{NO}_2$ , foliar  $\delta^{15}\text{N}$ , and soil  $\delta^{15}\text{N}$  values along a highway in Switzerland. This study showed that leaves of plants growing in close proximity to an emission source reflected the  $\delta^{15}\text{N}$  values of the N pollution (Ammann *et al.* 1999). Specifically, the foliar  $\delta^{15}\text{N}$  values increased from -3.7‰ to -1.5‰ within one growing season. Total anthropogenic inputs of both soil-derived N (-3.7‰) and gaseous  $\text{NO}_2$  (5.7‰) contributed about 25% to the total N budget of vegetation adjacent to the emissions source. However, the experimental methodology did not allow for the separation of soil vs. foliar uptake sources of anthropogenic N. Additionally, whether the differences in  $\delta^{15}\text{N}$  are due to differential soil cycling, fractionations within the plant, or foliar uptake is unknown. It is critical that future research address the importance and resulting impacts of these fundamentally different modes of anthropogenic N uptake to terrestrial ecosystems.

### **Future research**

Foliar  $\delta^{15}\text{N}$  measurements have the potential to be powerful indicators of the relative contribution of soil vs. foliar uptake of atmospheric reactive N inputs to terrestrial ecosystems in both rural and urban environments. Natural

abundance foliar  $\delta^{15}\text{N}$  measurements provide us with an integration of the  $\delta^{15}\text{N}$  of the N sources and fractionation events that occur during foliar N uptake, allocation, and emissions of N compounds from vegetation. However, the current understanding of sources and fractionation events is incomplete and using foliar  $\delta^{15}\text{N}$  as a tool to quantify a single process, like foliar uptake, is currently challenging. Although recent modeling efforts have made significant headway in identifying processes that influence foliar  $\delta^{15}\text{N}$  (Comstock 2001), no work has examined the fractionation events explicitly associated with foliar N uptake. For foliar  $\delta^{15}\text{N}$  measurements to become an effective research tool in determining foliar uptake of N pollution, we must further develop models for  $^{15}\text{N}$  uptake and discrimination by leaves to a point analogous to those developed for plant carbon isotope composition where all sources and fractionation events are considered (Evans 2001, Farquhar *et al.* 1982).

Additionally, the use of dual isotope methods promises to be a useful tool to investigate the impact of reactive N exposure; not only foliar uptake of atmospheric N pollution, but also as a way to analyze changes in physiology, morphology and carbon partitioning in vegetation exposed to atmospheric reactive N sources (Durka *et al.* 1994, Siegwolf *et al.* 2001). Recent advances in methodology using  $^{13}\text{C}/^{15}\text{N}$  and  $^{18}\text{O}/^{15}\text{N}$  dual isotope approaches (Cernusak *et al.* 2002, Gaudinski *et al.* 2005, McIlvin & Casciotti 2006) provide great potential to expand our knowledge of N source partitioning and fractionation events along multiple spatial and temporal scales. In particular, natural abundance ratios of  $^{18}\text{O}/^{16}\text{O}$  in atmospheric  $\text{NO}_3^-$  sources differ from those of nitrification derived  $\text{NO}_3^-$  and may be a powerful diagnostic tool. Together with  $^{15}\text{N}/^{14}\text{N}$ , stable oxygen isotope analysis of leaf water could be very useful in

determining the fate of anthropogenic N sources to vegetation. This method has successfully been applied to identifying anthropogenic N sources to stream water (Piatek *et al.* 2005), but has not yet been applied to plant N dynamics, an area ripe for further investigation.

Laboratory based hydroponics-fumigation systems provide a foundation to test and verify our current understanding of the foliar uptake of pollutant N and how this additional source of reactive N to plants will affect the plant N budget. It may be possible to use a parallel strategy to that applied to hydroponics-fumigation systems in the field. Examining the divergence between natural abundance foliar  $\delta^{15}\text{N}$  and the  $\delta^{15}\text{N}$  of extractable  $\text{NO}_3^-/\text{NH}_4^+$  (plant-available soil N) along a gradient of anthropogenic N deposition will assess whether the difference in magnitude between these measurements reflects the uptake of atmospheric N pollution directly via the leaves. Any such effort will need to account for the influence of mycorrhizae and verify the methodology of accurately quantifying soil solution  $\delta^{15}\text{N}$ .

Currently, there are no published experiments that have directly quantified the proportion of N assimilated through foliage or quantitatively demonstrated a positive relationship between foliar N uptake and anthropogenic N pollution in natural systems. Clearly, as atmospheric reactive N deposition increases across the globe, we need to develop effective methods to identify and trace the resulting ecological change due to human-mediated additions of reactive N. Future efforts must include both estimates of total N deposition and archives of foliar  $\delta^{15}\text{N}$  values across a variety of ecosystems.

## Conclusions

This chapter highlights the importance and utility of the foliar  $\delta^{15}\text{N}$  value as a tool for determining the magnitude and impact of atmospheric N pollution directly entering the foliage of vegetation. Substantial progress has been made in furthering our understanding of the mechanisms and environmental factors that control foliar  $\delta^{15}\text{N}$  and the contribution of foliar uptake of anthropogenic N pollution to individual plant N budgets. Recent hydroponics-fumigation studies have successfully used foliar  $\delta^{15}\text{N}$  as a tool for investigating and quantifying the proportion of foliar uptake of atmospheric N pollution. *In situ* coupled measurements of natural abundance foliar and soil solution  $\delta^{15}\text{N}$  across a range of ecosystems and species will shed further light on the validity of foliar  $\delta^{15}\text{N}$  as an accurate indicator of ecological change in plant N dynamics. A firm understanding of both the magnitude of foliar N uptake and whether foliar  $^{15}\text{N}$  values may be used as a tracer to quantify pollutant N assimilation is imperative for understanding the future global N cycle.

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CHAPTER 2:  
QUANTIFYING FOLIAR UPTAKE OF GASEOUS NITROGEN DIOXIDE  
USING ENRICHED FOLIAR  $\delta^{15}\text{N}$  VALUES\*

**Abstract**

The magnitude and impact of gaseous nitrogen dioxide ( $\text{NO}_2$ ) directly entering the leaves were investigated using foliar  $\delta^{15}\text{N}$  values in tomato (*Lycopersicon esculentum*) and tobacco (*Nicotiana tabacum*). Using a hydroponics-fumigation system,  $^{15}\text{NO}_2$  (20 ppb and 40 ppb) was supplied to shoot systems and  $\text{NO}_3^-$  (50  $\mu\text{M}$  and 500 $\mu\text{M}$ ) was supplied to root systems. Morphological, stable isotope and nitrate reductase activity (NRA) analyses were used to quantify foliar  $\text{NO}_2$  uptake and examine whether realistic concentrations of  $\text{NO}_2$  influenced plant metabolism. *Nicotiana tabacum* and *L. esculentum* incorporated 15 and 11%, respectively, of  $^{15}\text{NO}_2\text{-N}$  into total biomass via foliar uptake under low  $\text{NO}_3^-$  supply. On a mass basis, *N. tabacum* and *L. esculentum* incorporated  $3.3 \pm 0.9$  and  $3.1 \pm 0.8$  mg of  $^{15}\text{NO}_2\text{-N}$  into biomass, respectively, regardless of  $\text{NO}_3^-$  availability. There were no strong effects on biomass accumulation or allocation, leaf  $\delta^{13}\text{C}$  values, or leaf and root NRA in response to  $\text{NO}_2$  exposure. Foliar  $\text{NO}_2$  uptake may contribute a significant amount of N to plant metabolism under N-limited conditions, does not strongly influence growth at 40 ppb, and may be traced using foliar  $\delta^{15}\text{N}$  values.

\*Vallano, D.M. and J.P. Sparks. 2008. Quantifying foliar uptake of gaseous nitrogen dioxide using enriched foliar  $\delta^{15}\text{N}$  values. *New Phytologist* 177(4): 946-955.



## Introduction

Nitrogen (N) deposition to ecosystems is potentially both an important source of nutrients to vegetation and a detriment to ecosystem function (Vitousek *et al.* 1997). The dramatic increase in atmospheric concentrations of reactive N oxides ( $\text{NO}_y$ ) and ammonia gases ( $\text{NH}_x$ ) from human activities has increased the input of N deposition to plants and soil (Gessler *et al.* 2002, Morikawa *et al.* 1998). These pollutant compounds are deposited to and incorporated into vegetation via two interrelated pathways: directly through the leaves and indirectly through the soil. In N-limited ecosystems, atmospheric reactive N compounds may represent a considerable source of N to vegetation and may have a significant impact on plant metabolism (Hill 1971, Siegwolf *et al.* 2001, Sparks *et al.* 2001).

Although numerous studies have shown the significant impacts of total N deposition on ecosystem processes (e.g. Aber *et al.* 2003, Galloway *et al.* 2003, Lovett 1994), few have investigated the amount of atmospheric reactive N incorporated directly by leaves. If foliar N uptake has a differential influence on plant metabolism compared with increased soil N availability, then understanding the source N partitioning between these two pathways will be necessary for predicting future ecosystem function.

The traditional view is that plants meet their N demand exclusively by root uptake of ammonium ( $\text{NH}_4^+$ ), nitrate ( $\text{NO}_3^-$ ), and dissolved organic N from the soil. However, plants are capable of incorporating atmospheric reactive N compounds, including  $\text{NO}_y$  and  $\text{NH}_x$ , directly through the leaves and

assimilating this N into plant metabolism (Hereid & Monson 2001, Sparks *et al.* 2001, Vallano & Sparks 2007). The influence of N deposition on the root uptake pathway in isolation and the simultaneous uptake through leaves and roots has been well documented (Collier *et al.* 2003, Gessler *et al.* 1998, Muller *et al.* 1996). However, independently quantifying foliar N uptake and determining if this N is assimilated into biomass have remained challenging. In this study, I used N stable isotopes to independently quantify uptake and assimilation through leaves at realistic atmospheric N concentrations.

The ability of vegetation to incorporate N into metabolism from both leaves and roots suggests that the fate of N deposited to an ecosystem may differ fundamentally depending upon the entry pathway. For example, the chemical products of foliar N assimilation are nutrients and are directly incorporated from the leaf apoplast and used for growth (Gessler *et al.* 2000, Morikawa *et al.* 1998). By contrast, N soil additions during deposition may be incorporated into microbial biomass, volatilized, or leached. Therefore, the direct entry of N into metabolism through leaves has the potential to fundamentally change plant productivity through a direct influence on photosynthesis. However, it is unclear if, and how, plant performance is differentially affected by changes in N deposition to the soil versus changes in N additions directly to leaves. Both forms of N entry into an ecosystem will likely increase under most scenarios of intensified industrialization. However, various gaseous reactive N forms have significantly different atmospheric lifetimes, suggesting that plant communities arrayed across the landscape experience not only unequal N deposition, but different chemical species depending upon the distance from a reactive N source.

Some evidence suggests that the proportion of N incorporated through the foliar versus soil pathway may vary across the environment. For example, atmospheric reactive N is dominated by relatively reduced forms (e.g. NO<sub>2</sub>) near industrial sources. These compounds deposit more readily through the stomata than to other surfaces in the environment (Sparks *et al.* 2001). At distances further from N pollution sources, much of the reactive N is oxidized to organic forms such as peroxyacetyl nitrate. These compounds also readily enter plant stomata (Sparks *et al.* 2003) at a rate higher than that of deposition to other surfaces. In regions still further from reactive N sources, the atmosphere is often dominated by nitric acid (HNO<sub>3</sub>). HNO<sub>3</sub> is soluble in water and tends to deposit to many surfaces in the environment at a faster rate than entry through the stomata (Byterowicz *et al.* 1998). As the composition of atmospheric reactive N compounds can vary across the landscape, with the result that they will be more or less likely to enter stomata, ecosystems may receive differential amounts of N through the foliar uptake pathway.

Nitrogen stable isotope ratios (<sup>15</sup>N:<sup>14</sup>N) are a potential tool for investigating N sources to plant metabolism (Robinson *et al.* 1998, Robinson *et al.* 2000), estimating the amount of atmospheric reactive N in a system, and identifying the uptake pathway into the plant. For instance, N sources available to plants often have varying N isotopic composition (δ<sup>15</sup>N) values (Kolb & Evans 2002). Foliar δ<sup>15</sup>N values may reflect the isotopic value of the N sources (Stewart *et al.* 2002). Therefore, the relative importance of various N sources for the plant N budget can be determined at the leaf level using N stable isotopes (Ammann *et al.* 1999). However, the variation in source δ<sup>15</sup>N values and an incomplete understanding of fractionation events make it challenging to

interpret foliar  $\delta^{15}\text{N}$  values in natural systems because it is difficult to distinguish the  $\delta^{15}\text{N}$  values of multiple N sources (Evans 2001). One potential strategy for accurately quantifying foliar N uptake is to use a single N source enriched in  $\delta^{15}\text{N}$  as a tracer by enhancing the magnitude of differences in  $\delta^{15}\text{N}$  in the various N pools available to the plant in a controlled laboratory system. This strategy ameliorates many of the interpretation problems caused by fractionation among N pools by creating a large signal separation and is useful for following the movement of N through a plant and for following the movement of N through a plant and for determining rates of N assimilation (Dawson *et al.* 2002).

In this study, a hydroponics-fumigation system was used with enriched  $^{15}\text{NO}_2$ , a common gaseous reactive N pollutant, as a tracer to create a separation in  $\delta^{15}\text{N}$  between N sources and to track foliar uptake and assimilation of  $\text{NO}_2\text{-N}$  through plants. This system allowed me to simultaneously and independently control the forms, concentrations, and  $\delta^{15}\text{N}$  of the N sources. Using this strategy, I focused on three questions:

1. What proportion and amount of N can be attributed to direct foliar uptake of  $\text{NO}_2$ ?
2. How is this response influenced by  $\text{NO}_3^-$  availability through the root system?
3. Is there variation in foliar N assimilation among species, as indicated by differences in  $\text{NO}_3^-$  reduction location and activity?

The goals of this study were to quantify the magnitude of plant tissue N originating from foliar uptake and to begin to explore the physiological mechanisms underlying species-level differences in foliar uptake capacity. I hypothesized that, at realistic levels of atmospheric NO<sub>2</sub> exposure, a significant amount of N entering metabolism could come from direct foliar uptake. Further, I expected that increased NO<sub>3</sub><sup>-</sup> availability to roots would diminish the magnitude of foliar NO<sub>2</sub> uptake and the impact of this pathway on N metabolism. Finally, I predicted variation in foliar N assimilation as a result of differences in NO<sub>3</sub><sup>-</sup> reduction strategies among species.

## **Materials and Methods**

### *Plants and treatments*

Tomato (*Lycopersicon esculentum* Mill.) and tobacco (*Nicotiana tabacum* L.) seeds were purchased from a plant producer (Paramount Seeds, Inc., Palm City, FL, USA). Tomato and tobacco were selected for this study because both are fast-growing, herbaceous species that tend to express nitrate reductase differentially in the plant body, and grow well in hydroponics systems. Seeds of both species ( $n = 36$  per species) were germinated and seedlings were grown in pots in perlite (Sun-Gro Horticulture, Bellevue, WA, USA) in a climate-controlled growth chamber (EGC, Chagrin Falls, OH, USA) at day and night temperatures of 27 and 21°C, respectively, under moderate light (500  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) and a 16-h photoperiod. Plants were watered daily to saturation for 2 weeks and fertilized with a Hoagland solution including NO<sub>3</sub><sup>-</sup> ( $\delta^{15}\text{N} = -2.7\text{‰}$ ). Twenty-four 2-week-old plants were transplanted into the hydroponics-

fumigation system and used for measurements. The remaining 12 plants per species were harvested and analyzed for biomass, morphology, and isotopic composition to provide a baseline for the experimental plants.

The hydroponics-fumigation system consisted of four 50-L opaque polyethylene nutrient tanks (120 x 58 x 16 cm) each fitted with three separate airtight Plexiglas fumigation chambers (36 x 25 x 43 cm) with an opaque base. The system was located in a glasshouse with day and night temperatures of 27 and 21°C, respectively, relative humidity of 60-70%, and moderate light conditions ( $800 \pm 75 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) using natural and supplemental metal halide lighting (400 MH Econo Cool Grow Light; Sunlight Supply, Vancouver, WA, USA) under a 16-h photoperiod. Plants were transplanted into the system with roots suspended in nutrient solution (20°C) in a hydroponics tank and the shoots enclosed in a fumigation chamber (ambient temperature). Each plant was stabilized and fitted with modeling clay (Loctite; Henkel Consumer Adhesives, Avon, OH, USA) at the root-shoot junction at the base of each chamber, to ensure an impermeable seal between the fumigation and nutrient solution system components.

Two N regimes (50 and 500  $\mu\text{M}$ ) were applied to the roots using nutrient solutions with fixed concentrations of  $\text{NO}_3^-$  as the sole root N source in a modified quarter-strength Hoagland's solution (Smart & Bloom 1993). The difference in concentration between the low-N (LN; 50  $\mu\text{M}$ ) and high-N (HN; 500  $\mu\text{M}$ ) regimes simulated N-limited and N-surplus conditions, respectively. The mean concentrations for the LN and HN regimes were  $46 \pm 2$  and  $481 \pm 28 \mu\text{M}$ , respectively. The  $\delta^{15}\text{N}$  of the nutrient solution was  $-2.7 \pm 0.2\text{‰}$ . The

nutrient solution was vigorously aerated at all times to provide adequate oxygenation and to ensure complete mixing. Solution pH was maintained at 5.8-6.2 by the addition of either KOH or H<sub>2</sub>SO<sub>4</sub>. A 130-L reservoir of stock solution was used for each experimental NO<sub>3</sub><sup>-</sup> treatment. Nutrient solutions were replaced weekly to minimize microbial activity and to prevent N depletion. The nitrate concentration was measured weekly using an automatic auto-analyzer (Astoria Pacific, Inc., Clackamas, OR, USA).

Fumigation chambers were supplied with filtered (using activated charcoal) ambient air using a reciprocating air compressor (Model C403L; Gardner Denver, Quincy, IL, USA) at a flow rate of 15 L min<sup>-1</sup>. Enriched <sup>15</sup>NO<sub>2</sub> from a compressed tank (1% NO<sub>2</sub> : 99% N<sub>2</sub>; Scott Marrin, Inc., Riverside, CA, USA) was diluted into the filtered air of selected chambers using high-precision rotometers and mass flow controllers (Models 03216-34 and 32044-00; Cole-Parmer, Vernon Hills, IL, USA) at fixed partial pressures (20 and 40 ppb). The δ<sup>15</sup>N of the enriched <sup>15</sup>NO<sub>2</sub> was 1720 ± 17‰, providing a large signal separation from the NO<sub>3</sub><sup>-</sup> source. NO<sub>2</sub> was selected as the atmospheric N source because it is a common atmospheric reactive N compound.

Treatments were selected to simulate suburban (20 ppb) and urban (40 ppb) atmospheric NO<sub>2</sub> conditions. Four randomly selected chambers were selected as controls and were not exposed to NO<sub>2</sub> fumigation (0 ppb). Therefore, there were six treatments: (1) 0 ppb NO<sub>2</sub> and 50 μM NO<sub>3</sub><sup>-</sup>, (2) 20 ppb NO<sub>2</sub> and 50 μM NO<sub>3</sub><sup>-</sup>, (3) 40 ppb NO<sub>2</sub> and 50 μM NO<sub>3</sub><sup>-</sup>, (4) 0ppb NO<sub>2</sub> and 500 μM NO<sub>3</sub><sup>-</sup>, (5) 20 ppb NO<sub>2</sub> and 500 μM NO<sub>3</sub><sup>-</sup>, and (6) 40 ppb NO<sub>2</sub> and 500 μM NO<sub>3</sub><sup>-</sup>. Within each treatment, there were two chambers with four plants per chamber (*n* = 8). NO<sub>2</sub> and NO concentrations were monitored using a chemiluminescence NO-

NO<sub>2</sub>-NO analyzer (TECO Model 42; Thermo Environmental Instruments, Inc., Franklin, MA, USA). Non-incorporated NO<sub>2</sub> was filtered using activated charcoal and exited the system through an output line extending outside the glasshouse. Temperature and relative humidity within the chambers were monitored using humidity and temperature probes (Model HMP45A; Vaisala, Inc., Boulder, CO, USA) connected to a datalogger (Model CR10x; Campbell Scientific, Inc., Logan, UT, USA).

#### *Morphological and stable isotope analysis*

Plants were harvested 28 d after the start of fumigation (or at the beginning of the experiment for baseline plants) and fresh tissue was weighed using a balance (Model M-2200; Denver Instrument, Denver, CO, USA). Whole-plant samples were separated into leaf, shoot, and root tissue. Shoot length and leaf number were measured for each individual and leaf area was estimated using a leaf area meter (LI-3100 Area Meter; Li-Cor, Inc., Lincoln, NE, USA).

After fresh weight morphological analyses, plant samples were dried and weighed, and subsamples were analyzed for tissue N and C contents and  $\delta^{15}\text{N}$ . Previously separated tissues samples were rinsed with deionized water to remove any NO<sub>2</sub> deposited to the leaf surface and dried for 72 h at 55°C in a drying oven. The dried tissue samples were weighed and ground to a fine powder with a mortar and pestle, and sub-samples of 2.55-3.15 mg were weighed using a microbalance (Model 4504MP8; Sartorius Corp., Edgewood, NY, USA). Tissue N and C contents were measured using a CHN elemental analyzer (Model Carlo Erba NC2500; Thermo Finnigan, San Jose, CA, USA).



Tissue  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  were measured using a continuous flow isotope ratio mass spectrometer (Model Delta Plus; Thermo Finnigan, San Jose, CA, USA). All analyses were conducted at the Stable Isotope Laboratory at Cornell University.

#### *Nitrate reductase assay*

Nitrate reductase activity (NRA) was assayed *in vitro* on frozen leaf and root tissues from eight seedlings per treatment according to Scheible *et al.* (1997). NRA was determined as the rate of  $\text{NO}_2^-$  accumulation and was measured at 540 nm using a spectrophotometer (DU 640; Beckman, Fullerton, CA, USA). The samples were compared with a standard curve generated using a  $\text{KNO}_2$  stock solution.

#### *Calculation of N source partitioning*

Partitioning of plant N among sources (gaseous  $\text{NO}_2$  and nutrient solution  $\text{NO}_3^-$ ) was calculated using a two-ended linear mixing model (Dawson *et al.* 2002) and published fractionation factors for root  $\text{NO}_3^-$  assimilation (Kolb & Evans 2003). Because we used an artificially high enrichment of  $^{15}\text{N}$  in the  $\text{NO}_2$ , fractionation events associated with leaf uptake were not detectable (i.e. the signal separation generated by the tracer was larger than all natural fractionations). Using this model, we can define the total amount of  $\text{NO}_2$  incorporated via direct foliar uptake during the fumigation period.

### *Statistical analysis*

Data represent three separate experiments conducted within a single year, each time using a new set of experimental plants (20 September to 19 October 2005, 25 January to 20 February 2006, and 18 May to 13 June 2006). No significant differences were detected among experiments, and, for simplicity, the stable isotope and morphological data were pooled across experimental runs. Data were statistically evaluated using a multifactorial ANOVA, with  $\text{NO}_3^-$  in the nutrient solution and  $\text{NO}_2$  fumigation treatments as the two factors. The experimental design included six total treatments and twelve fumigation chambers, resulting in two replicates per treatment per experiment. Statistical analysis was carried out using data from three experimental runs, resulting in means calculated from a total of six fumigation chambers. Significance levels for all tests were  $P \leq 0.05$ . Statistical analyses were performed using SAS software (Version 9.0) and SAS JMP software (Version 3.2.5; SAS Institute, Cary, NC, USA).

## **Results**

### *Foliar uptake and N source partitioning*

Both species incorporated N via foliar uptake into total biomass under  $\text{NO}_2$  fumigation (Fig. 2.1a,b). For both *L. esculentum* and *N. tabacum*, there was a significant proportion of N incorporated via foliar uptake in individuals exposed to  $\text{NO}_2$  regardless of  $\text{NO}_3^-$  supply ( $P < 0.05$ ; Fig. 2.1a). Regardless of species, the highest proportion of N incorporated via foliar uptake was observed under

low  $\text{NO}_3^-$  (LN) conditions and the highest  $\text{NO}_2$  fumigation treatment (40 ppb  $\text{NO}_2$ ). Under this treatment, *L. esculentum* and *N. tabacum* incorporated  $11.0 \pm 0.9$  and  $14.9 \pm 0.8\%$  of total plant N through foliar uptake. The  $\delta^{15}\text{N}$  values

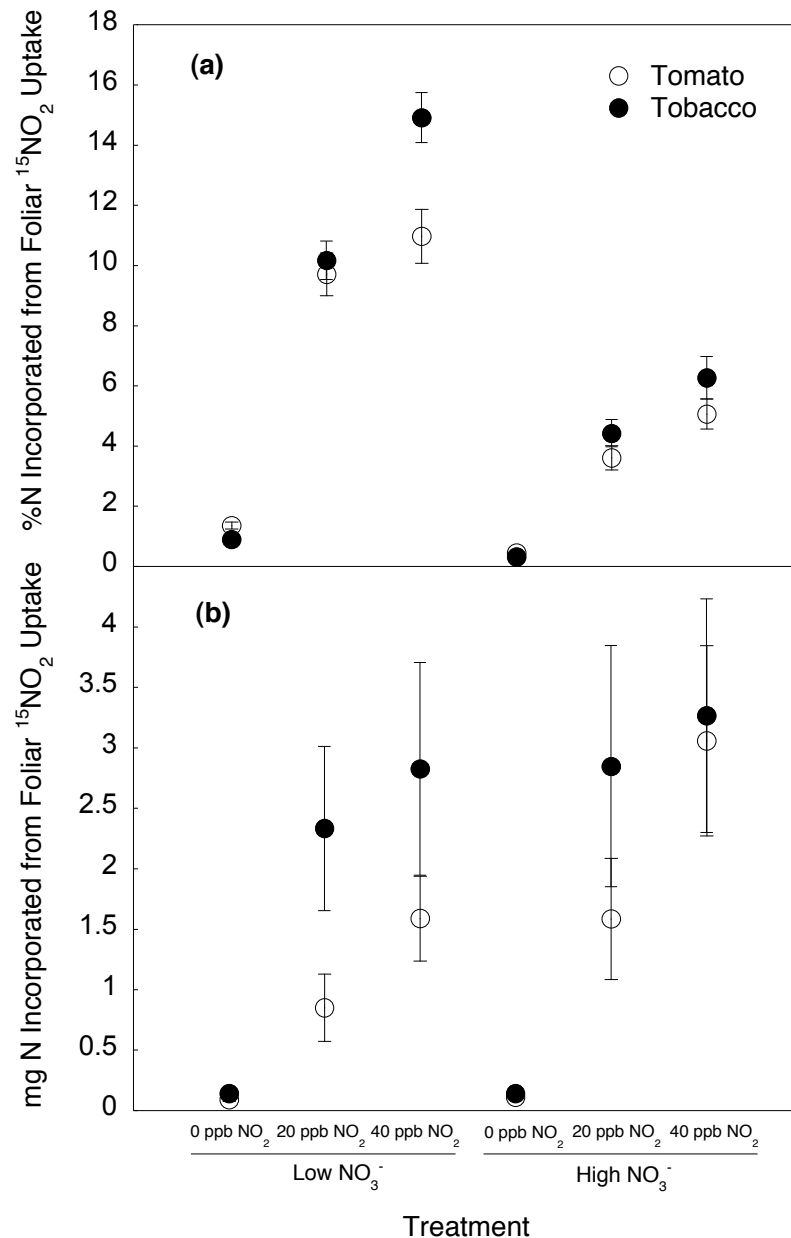


Figure 2.1. Proportion (%) (a), and amount (in mg) (b) of total N content attributable to the direct foliar uptake of  $^{15}\text{NO}_2\text{-N}$  in *N. tabacum* (●) and *L. esculentum* (○) for each treatment. Data are means  $\pm 1$  standard error ( $n = 16$  for *L. esculentum*;  $n = 12$  for *N. tabacum*). Significance level was set at  $P \leq 0.05$ . An asterisk indicates significant differences between species.

for *L. esculentum* and *N. tabacum* under this treatment were  $188.5 \pm 15.5\%$  and  $257.0 \pm 14.6\%$ , respectively. Individuals of both species fumigated with  $\text{NO}_2$  and grown under LN conditions incorporated a significantly higher proportion of N via foliar uptake compared with individuals grown under high  $\text{NO}_3^-$  (HN) ( $P < 0.05$ ). We observed significant differences in foliar  $\text{NO}_2$ -N uptake between the two different  $\text{NO}_2$  concentrations (20 and 40 ppb) under LN conditions in *N. tabacum* ( $P = 0.0064$ ) but not under HN conditions. We did not observe any such differences for *L. esculentum* regardless of  $\text{NO}_3^-$  availability. Species differences were only observed in individuals exposed to 40 ppb  $\text{NO}_2$  under LN conditions. Under this treatment, *N. tabacum* incorporated 4% more N from foliar uptake than *L. esculentum* ( $P = 0.003$ ).

When examined on a mass rather than a proportion basis (Fig. 2.1b), *L. esculentum* and *N. tabacum* incorporated  $3.1 \pm 0.8$  and  $3.3 \pm 0.9$  mg of  $\text{NO}_2$ -N, respectively. In contrast to the proportional measurements, the total mass of  $\text{NO}_2$ -N incorporated into plant biomass via foliar uptake was similar regardless of root  $\text{NO}_3^-$  conditions. Furthermore, there were no differences in the amount of N incorporated via foliar uptake between  $\text{NO}_2$  fumigation levels (20 vs 40 ppb) for either species. There were also no significant species differences in the amount of N incorporated from foliar uptake.

The amount of N derived from foliar uptake of  $\text{NO}_2$  in both species varied among root, shoot, and leaf tissues (Fig. 2.2a,b). In both species, the amount of total N incorporated via foliar uptake was significantly higher in leaf tissue compared with root and shoot tissues. In fumigated individuals of *N. tabacum*, leaf tissue contained 78–82% more N (in mg) from foliar  $\text{NO}_2$  uptake than root

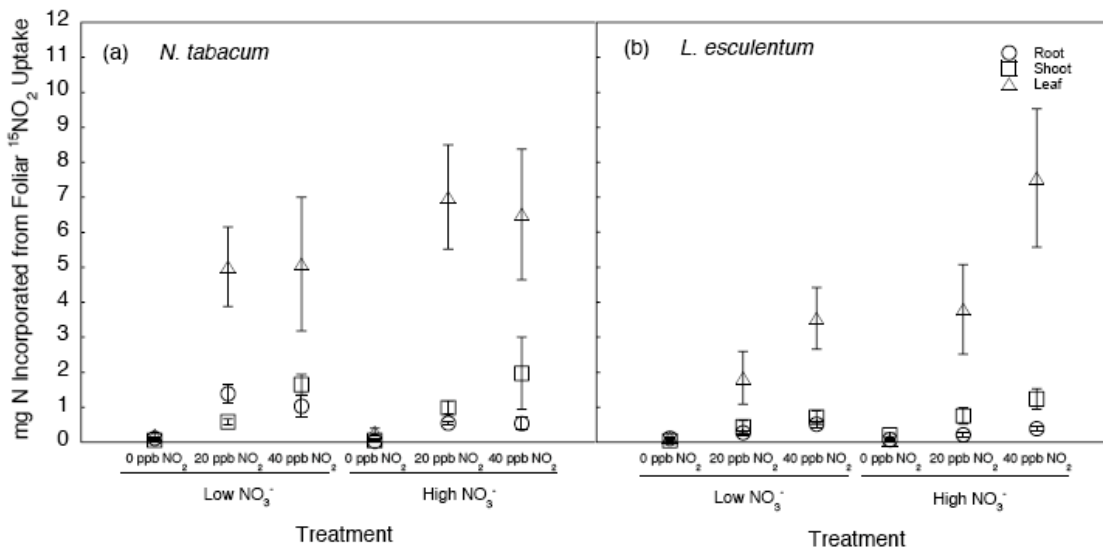


Figure 2.2. Amount (in mg) of total nitrogen attributable to the direct foliar uptake of  $^{15}\text{NO}_2\text{-N}$  in *N. tabacum* (a) and *L. esculentum* (b) for root ( $\circ$ ), shoot ( $\square$ ), and leaf ( $\triangle$ ) tissue for each treatment. Data are means  $\pm$  1 standard error ( $n = 16$  for *L. esculentum*;  $n = 12$  for *N. tabacum*).

and shoot tissues (Fig. 2.2a). In fumigated individuals of *L. esculentum*, leaf tissue contained 69-93% more N (in mg) from the foliar  $\text{NO}_2$  uptake than root and shoot tissues (Fig. 2.2b). Similar to the total plant measurements, the total mass of  $\text{NO}_2\text{-N}$  incorporated into specific tissue types was not influenced by root  $\text{NO}_3^-$  conditions for either species.

#### *Morphology and biomass allocation*

Neither *L. esculentum* nor *N. tabacum* linearly increased total biomass accumulation (in g DW) in response to  $\text{NO}_2$  exposure (Fig. 2.3a). However, in *N. tabacum*,  $\text{NO}_2$  exposure increased biomass accumulation at 20 ppb  $\text{NO}_2$  under both LN and HN supply (by 55 and 92%, respectively), suggesting a fertilizer effect of  $\text{NO}_2$  exposure. Interestingly, individuals fumigated with 40

ppb NO<sub>2</sub> showed similar biomass accumulation to non-fumigated individuals. By contrast, NO<sub>2</sub> exposure decreased biomass accumulation at 20 ppb NO<sub>2</sub> in *L. esculentum* under LN and HN supply (by 59 and 32%, respectively), suggesting a toxic effect of NO<sub>2</sub> exposure. Similar to *N. tabacum*, individuals fumigated with 40 ppb NO<sub>2</sub> exhibited similar biomass accumulation compared with non-fumigated individuals (Fig. 2.3a). Species differences in total biomass across all treatments were only observed between individuals exposed to 20 ppb NO<sub>2</sub> under LN conditions. Under this treatment, *N. tabacum* incorporated 70% more total biomass than *L. esculentum* ( $P = 0.012$ ). Overall, no significant differences in growth were observed between experimental runs.

Both *L. esculentum* and *N. tabacum* exhibited significant differences in the ratio of root to shoot (stem + leaf) tissue in response to differences in root N supply. Further, *N. tabacum* also showed changes in biomass allocation in response to differences in NO<sub>2</sub> exposure under LN conditions ( $P < 0.05$ , Fig. 2.3b). In both species, higher NO<sub>3</sub><sup>-</sup> availability was accompanied by a change in whole-plant C partitioning, favoring shoot growth relative to root growth regardless of NO<sub>2</sub> fumigation concentration and resulting in a lower root:shoot ratio compared with individuals under LN availability ( $P < 0.05$ ). NO<sub>2</sub> exposure stimulated shoot relative to root growth in *N. tabacum*, causing a decrease (by 40%) in the root:shoot ratio in individuals exposed to 40 ppb NO<sub>2</sub> and LN conditions. However, NO<sub>2</sub> exposure did not affect root:shoot ratios in *esculentum*. A decrease in the root:shoot ratio in *N. tabacum* at 20 ppb NO<sub>2</sub> *L.* (by 14%) was also observed, but this difference was not significant. There were no significant species differences in biomass allocation observed for any treatments. Leaf area did not differ under any treatment for either species.

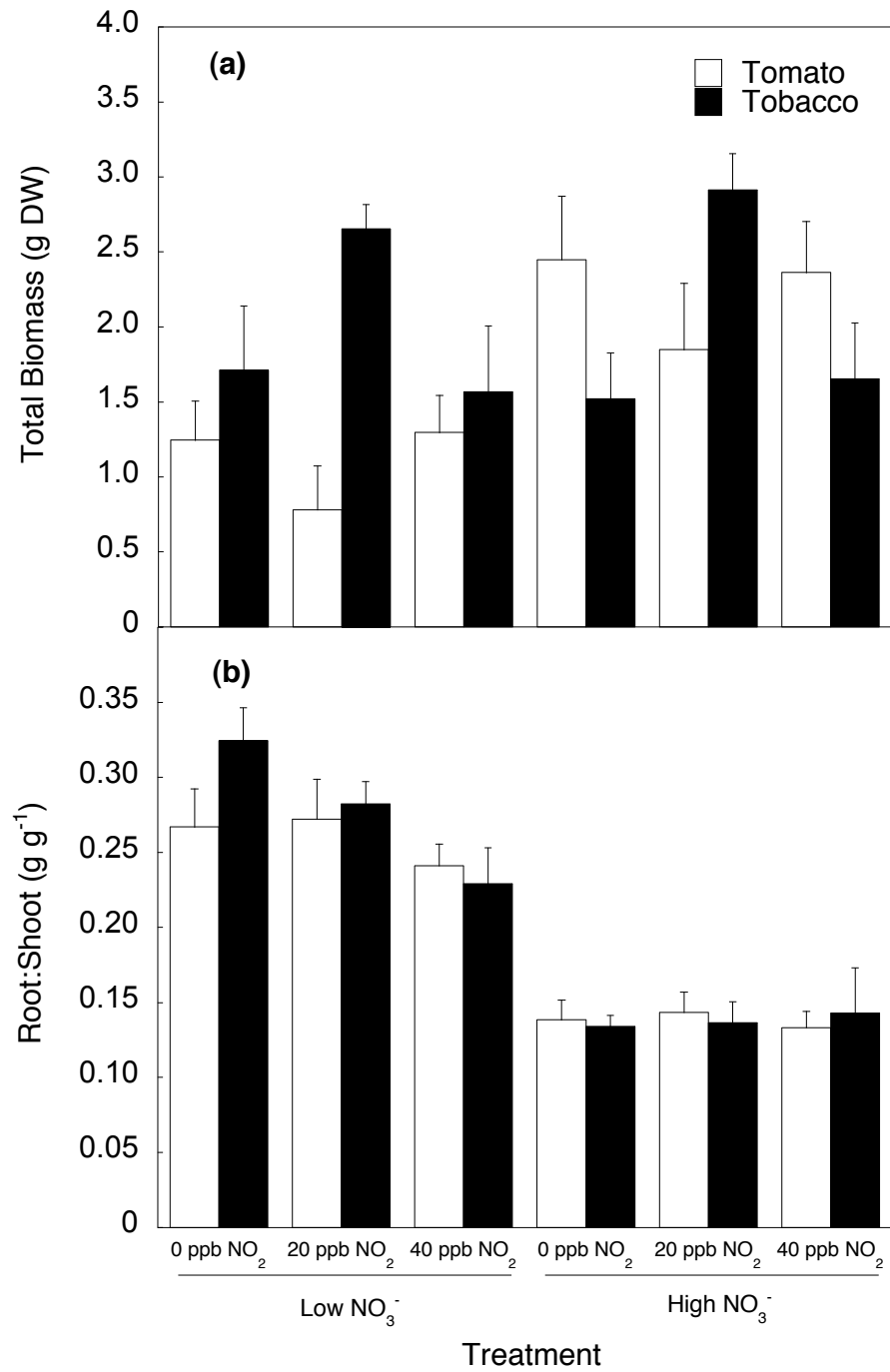


Figure 2.3. Total plant biomass (a) and root/shoot ratio (b) of *N. tabacum* (■) and *L. esculentum* (□) over all treatments. Data are means  $\pm$  1 SE ( $n = 16$  for *L. esculentum*;  $n = 12$  for *N. tabacum*). Significance level was set at  $P \leq 0.05$ . An asterisk indicates significant differences between species.

Differences in C and N allocation patterns were observed in response to differences in  $\text{NO}_3^-$  availability in *L. esculentum* and *N. tabacum*, but no effects of  $\text{NO}_2$  exposure were observed for either species (Fig. 2.4). Both species expressed lower C:N ratios under HN availability compared with LN availability ( $P < 0.05$ ). Overall, *N. tabacum* exhibited higher C:N ratios under LN supply

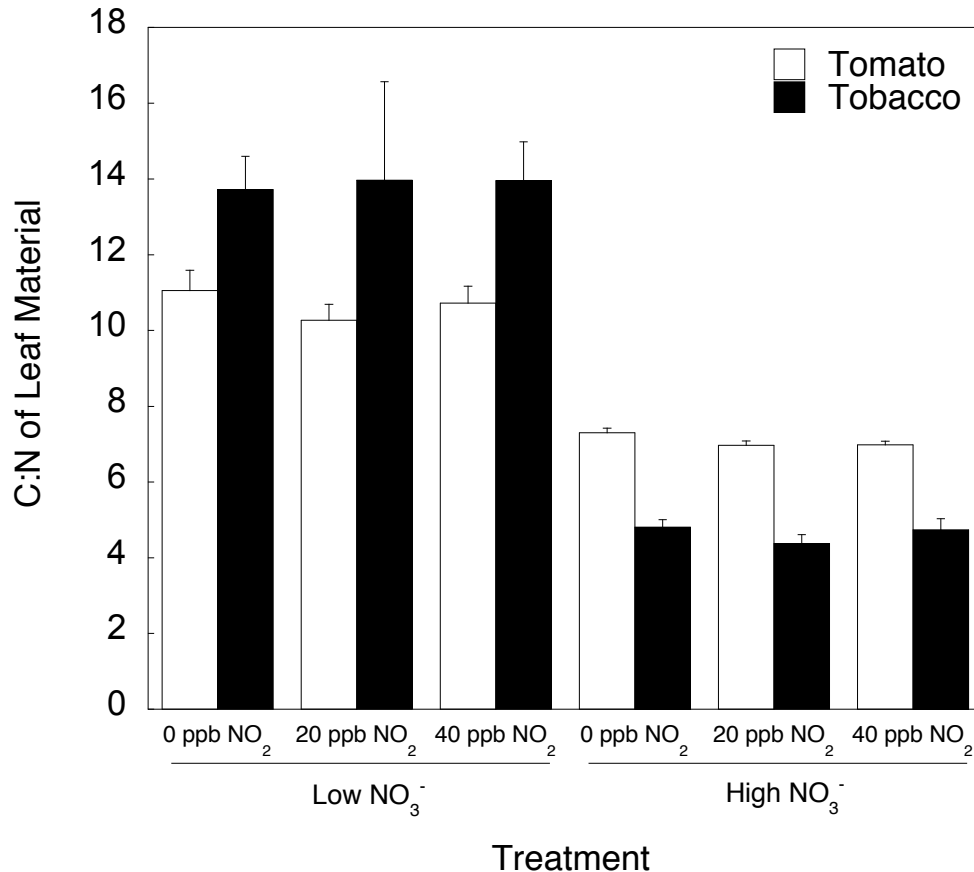


Figure 2.4. Carbon to nitrogen ratio for each treatment in *N. tabacum* (■) and *L. esculentum* (□). Data are means  $\pm 1$  SE ( $n = 16$  for *L. esculentum*;  $n = 12$  for *N. tabacum*). Significance level was set at  $P \leq 0.05$ . An asterisk indicates significant differences between species.

compared with *L. esculentum* and lower C:N ratios under HN supply compared with *L. esculentum* across all  $\text{NO}_2$  fumigation treatments. There was no within-species variation in C or N concentrations between  $\text{NO}_2$  fumigation levels regardless of  $\text{NO}_3^-$  supply for either species.



*L. esculentum* exhibited differences in the pattern of leaf  $\delta^{13}\text{C}$  in response to differences in  $\text{NO}_3^-$  availability ( $P < 0.05$ ), but *N. tabacum* did not exhibit any differences (Fig. 2.5). Neither species showed differences in leaf  $\delta^{13}\text{C}$

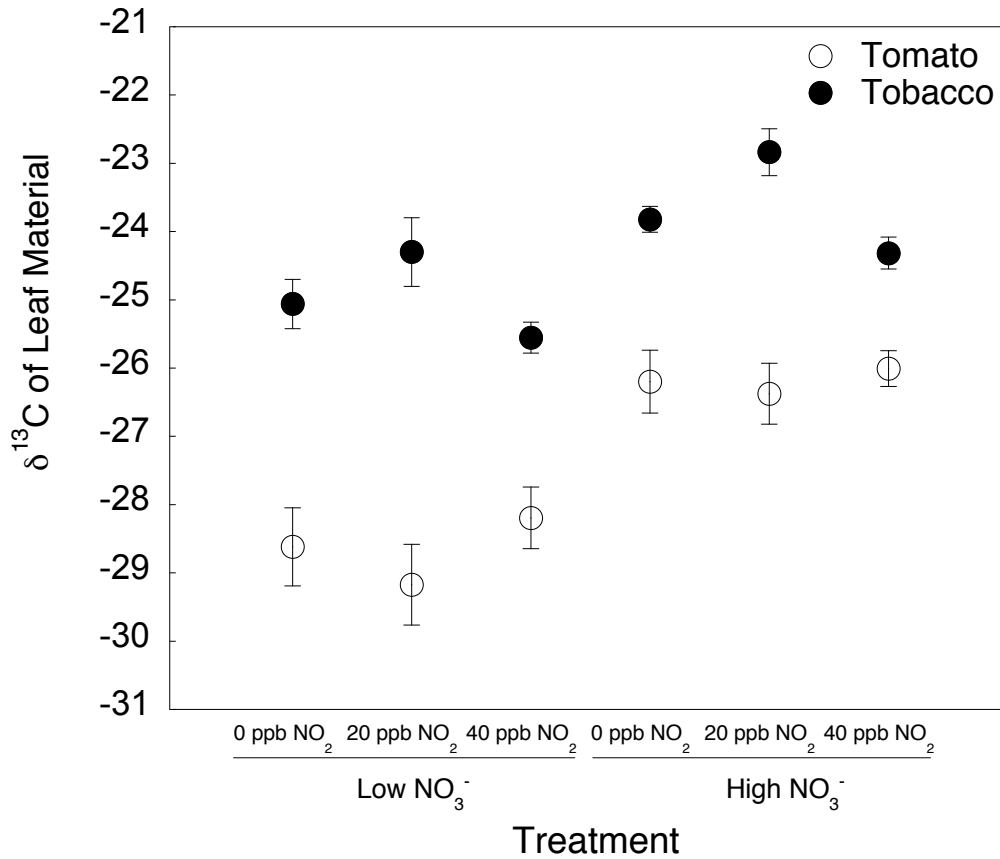


Figure 2.5. Foliar  $\delta^{13}\text{C}$  across treatments in *N. tabacum* (■) and *L. esculentum* (□). Data are means  $\pm$  1 SE ( $n = 16$  for *L. esculentum*;  $n = 12$  for *N. tabacum*). in response to differences in  $\text{NO}_2$  exposure. Species differences in leaf  $\delta^{13}\text{C}$  between *N. tabacum* and *L. esculentum* were observed for all treatments, with *N. tabacum* exhibiting higher leaf  $\delta^{13}\text{C}$  values than *L. esculentum* ( $P < 0.05$ ).

#### Nitrate reductase activity

There were no differences in leaf or root NRA in response to differences in

$\text{NO}_3^-$  availability or  $\text{NO}_2$  exposure in either species (Fig. 2.6a,b). Species differences in NRA were observed only in roots and only between individuals

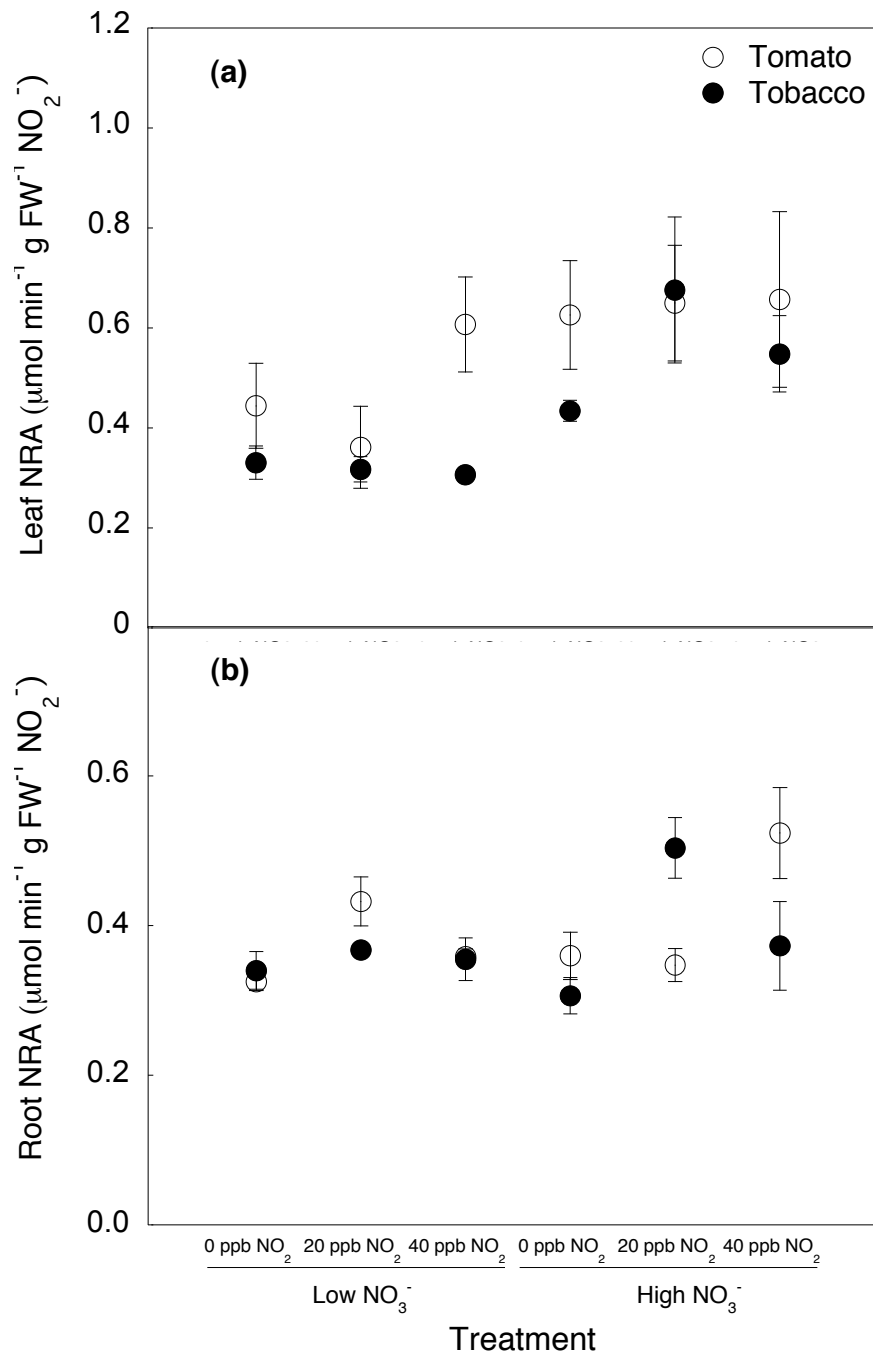


Figure 2.6. Leaf NRA (a) and root NRA (b) for each treatment in *N. tabacum* (□) and *L. esculentum* (●). Data are means  $\pm 1$  SE ( $n = 12$  for *L. esculentum*;  $n = 12$  for *N. tabacum*).

exposed to 20 ppb  $\text{NO}_2$  and HN supply ( $P = 0.018$ ). Under this treatment, *N. tabacum* showed higher root NRA than *L. esculentum* (Fig. 2.6b). Despite only small amounts of variation in NRA, we tested for a positive relationship between leaf NRA and the amount of  $\text{NO}_2\text{-N}$  incorporated via the foliar uptake pathway in both *L. esculentum* and *N. tabacum* (Fig. 2.7a,b). We observed a positive relationship between these parameters in *L. esculentum* ( $P = 0.019$ ), although the correlation was not statistically significant in *N. tabacum* ( $P = 0.312$ ).

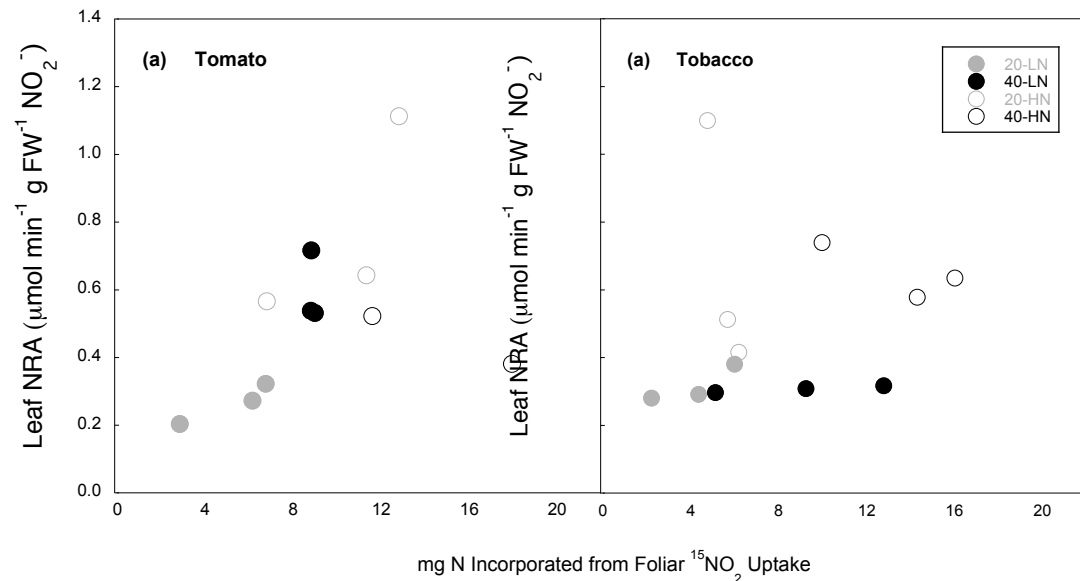


Figure 2.7. Leaf NRA vs. mg N incorporated from foliar  $^{15}\text{NO}_2$  uptake in *L. esculentum* (a) and *N. tabacum* (b) for each treatment. Data are means  $\pm$  1 SE ( $n = 12$  for *L. esculentum*;  $n = 12$  for *N. tabacum*).

## Discussion

The potential for foliar N uptake is established in the literature (Gessler *et al.* 1998, Muller *et al.* 1996, Takahashi *et al.* 2005). However, it remains challenging to independently quantify foliar uptake, rather than examining a

tracer applied simultaneously to leaves and soil, and to definitively determine if the N is assimilated into biomass. Further, most studies have evaluated foliar uptake at unrealistically high concentrations. The NO<sub>2</sub> fumigation concentrations used in this study (20 and 40 ppb) are relevant to ambient NO<sub>2</sub> exposure and trends. Currently, the United States Environmental Protection Agency Office of Air Quality Planning and Standards (OAQPS) has established a National Ambient Air Quality Standard for NO<sub>2</sub> pollution at 53 ppb. Additionally, the national average has steadily declined since 1980 to an average of ~20 ppb (EPA 2007).

We show that foliar uptake is a significant pathway of NO<sub>2</sub> entry and that the magnitude of foliar NO<sub>2</sub> uptake has the potential to influence plant metabolism. Further, there is some variation among species in their capacity to incorporate NO<sub>2</sub> via foliar uptake, an area of research that has yet to be thoroughly addressed (although see Morikawa *et al.* 1998). The average proportion of NO<sub>2</sub> incorporated via foliar uptake quantified in this study is similar in magnitude to values modeled for other plant species (Ammann *et al.* 1999, Muller *et al.* 1996). Specifically, Muller *et al.* (1996) estimated proportions of 10–15% in Norway spruce (*Picea abies*), Ammann *et al.* (1999) reported similar proportions of 10–25% in *P. abies*, and Siegwolf *et al.* (2001) estimated values in the range of 15–20% of total plant N in hybrid poplar (*Populus euramericana*) originating from NO<sub>2</sub> applied to both leaves and soil. However, these studies did not attempt to separate uptake pathways (soil versus foliar) and were likely complicated by many of the soil factors discussed in the Introduction. This study extends previous work by clearly

isolating and quantifying the direct impact of the foliar uptake pathway on plant metabolism.

We observed that foliar  $\text{NO}_2$  incorporation in both species and the magnitude of this uptake appeared to be related to soil N availability (Fig. 2.1a), perhaps suggesting that plants regulate the capacity for foliar uptake in response to changes in overall N availability. We also observed significant differences in foliar  $\text{NO}_2$ -N uptake between the  $\text{NO}_2$  concentrations (20 and 40 ppb) under LN conditions in *N. tabacum* ( $P = 0.0064$ ), suggesting that foliar uptake capacity in *N. tabacum* may be more sensitive to changes in  $\text{NO}_2$  concentrations than *L. esculentum*. However, when the incorporated foliar  $\text{NO}_2$ -N was examined on a mass basis (Fig. 2.1b), we observed similar total amounts of  $\text{NO}_2$ -N under both  $\text{NO}_3^-$  and  $\text{NO}_2$  treatments. Further, we observed no differences in the amount of incorporated foliar  $\text{NO}_2$ -N in individuals fumigated with 20 or 40 ppb  $\text{NO}_2$ , which may suggest that plants have a maximum threshold for the amount of N assimilated via foliar uptake. These results also indicate that foliar uptake rates are similar and any differences seen in the proportions of foliar uptake across  $\text{NO}_3^-$  treatments are likely driven by dilution into a larger plant N pool rather than an increase in foliar uptake rate in response to a decrease in soil N availability.

Nitrogen from foliar uptake was partitioned differentially across tissue components (i.e. leaf, shoot, and root) in both *L. esculentum* and *N. tabacum*. Not surprisingly, we observed a greater amount of  $\text{NO}_2$  incorporated into leaf tissue compared with roots and shoots under all treatments (Fig. 2.2a,b). This pattern of N assimilation suggests that most of the  $\text{NO}_2$ -N assimilated by the plant is directly incorporated into metabolism in the leaves and that there is

only limited reallocation of N to other plant organs. This pattern of N assimilation makes intuitive sense given the large sink for N in leaves and suggests a potential impact on plant productivity through a direct influence on photosynthesis.

No toxic effects on growth were observed, suggesting that the NO<sub>2</sub> concentrations applied were not detrimental to growth (Fig. 2.3). In fact, the biomass information suggests that NO<sub>2</sub> fumigation enhanced growth under some circumstances. For example, biomass production in *N. tabacum* increased by ~92% under the 20 ppb NO<sub>2</sub> and LN availability treatment. However, both species, when exposed to 40 ppb NO<sub>2</sub>, exhibited growth similar to that of control plants. This suggests that any beneficial fertilization effect occurs only at relatively low concentrations. The 40 ppb NO<sub>2</sub> fumigation did not appear to enhance or inhibit growth. This may mean that increased growth as a result of additional N availability is counteracted by increased toxicity, but this requires further evaluation across a wider range of fumigation levels. An NO<sub>2</sub>-driven influence on biomass allocation was observed in *N. tabacum* (Fig. 2.3b); a decrease in root:shoot ratio in response to an increase in NO<sub>2</sub> exposure under LN availability. This result is consistent with those of other studies (Muller *et al.* 1996, Siegwolf *et al.* 2001) that reported an increased C investment into leaves at high atmospheric N availability. This observation, as noted by others (Nussbaum *et al.* 1999, Siegwolf *et al.* 2001), suggests that a change in root:shoot ratio induced by NO<sub>2</sub> may have indirect consequences for growth and survival. For example, root:shoot ratio often varies across plants based on water and nutrient availability to balance water uptake and loss.

Therefore, changes in root:shoot ratio induced by NO<sub>2</sub> may negatively impact plant tolerance to drought or low nutrient conditions.

We examined the effects of NO<sub>2</sub> exposure on foliar  $\delta^{13}\text{C}$  values (Fig. 2.5) to explore the influence of exposure on the integrated ratio of external to internal CO<sub>2</sub> concentration ( $C_i:C$ ). Typically, a higher N supply can lead to increased stomatal conductance or higher leaf carboxylation capacity influencing the foliar  $\delta^{13}\text{C}$  value (Schulze *et al.* 1996). Across all treatments, we observed differences in foliar  $\delta^{13}\text{C}$  values in only *L. esculentum* in response to higher NO<sub>3</sub><sup>-</sup> availability, but not NO<sub>2</sub> exposure. Our results suggest that NO<sub>2</sub> exposure at these concentrations did not impact stomatal conductance or photosynthetic capacity.

Although the ability of vegetation to incorporate atmospheric reactive N via foliar uptake has been demonstrated, only a few studies have started to examine the variation in foliar N uptake capacity among species (e.g. Morikawa *et al.* 1998). In this study, we observed differences in patterns of biomass accumulation and the proportion of NO<sub>2</sub> incorporated through leaves between *L. esculentum* and *N. tabacum* with increasing NO<sub>2</sub> fumigation (Figs. 2.1, 2.3), reinforcing the idea that variation in foliar N uptake exists and may be influenced by species-specific properties. Diffusion processes and variation in stomatal dynamics among species are the primary drivers of variation in foliar N incorporation (Sparks *et al.* 2001, Weber & Rennenberg 1996). However, variation in foliar N uptake among species is likely controlled by factors in addition to diffusional resistance. For example, if stomatal conductance remains fairly constant, variations in the biochemical capacity for

NO<sub>2</sub> assimilation would alter the substomatal air cavity NO<sub>2</sub> concentration, and subsequently influence the NO<sub>2</sub> concentration gradient into the leaf and the rate of foliar NO<sub>2</sub> uptake (Hereid & Monson 2001). This ‘internal’ resistance to uptake is often termed the mesophyll resistance and has been suggested to be controlled by various factors, including the permeability of NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup> ions through cell membranes and walls (Ammann *et al.* 1995), the activity of ascorbic acid and other antioxidant compounds in the leaf apoplast (Teklemariam & Sparks 2004), and the site of activity in the primary assimilation NO<sub>3</sub><sup>-</sup> pathway through which NO<sub>2</sub> is (Wellburn 1990). To further investigate the role of the location of N reduction within the plant as a possible factor controlling the variation in foliar uptake capacity in *L. esculentum* and *N. tabacum*, we examined the relationship between leaf NRA and foliar NO<sub>2</sub> incorporation. The balance between root and shoot NO<sub>3</sub><sup>-</sup> reduction and the impacts of foliar NO<sub>2</sub> uptake on that balance, if variable among species, may strongly influence species responses to NO<sub>2</sub> exposure. It has been previously reported that *L. esculentum* and *N. tabacum* differ in the location of N reduction (Gojon *et al.* 1994). In *N. tabacum*, NO<sub>3</sub><sup>-</sup> is reduced into an organic form primarily in the shoot, while *L. esculentum* reduces NO<sub>3</sub><sup>-</sup> mainly in the roots. Previous work (as cited in Tischner 2000) has suggested that NRA stimulation will follow NO<sub>2</sub> exposure, although other studies have reported very little, if any, NRA stimulation in response to fumigation (Muller *et al.* 1996). We originally hypothesized that these species would differ significantly in leaf NRA activity and provided a system with which to examine differences in NO<sub>2</sub> uptake driven by differential metabolism. However, we observed no differences in leaf or root NRA between these two species regardless of NO<sub>2</sub> exposure, including controls (Fig. 2.6). Similarly, our results



did not show a correlation between leaf NRA and the NO<sub>2</sub> fumigation level in either species, indicating that leaf NRA was not stimulated by NO<sub>2</sub> exposure (Fig. 2.6). However, when the entire range of leaf NRA was considered (Fig. 2.7), we observed a positive relationship between leaf NRA and the amount of NO<sub>2</sub> incorporated via foliar uptake in *L. esculentum*, indicating the potential of leaf NRA stimulation in response to NO<sub>2</sub> exposure.

Foliar NO<sub>2</sub> uptake is significant in magnitude and can potentially influence plant metabolism and the plant N budget in terrestrial ecosystems impacted by atmospheric reactive N pollution. The results of this study give a quantitative estimate of the mass of NO<sub>2</sub> assimilated into biomass through the foliar uptake pathway. Obviously, hydroponics-fumigation experiments like the one we describe here are not an adequate simulation of natural conditions. However, they do provide significant insight into the transport of N pollutants into plants, and the physiological mechanisms that influence the capacity of various species to incorporate atmospheric reactive N via the foliar uptake pathway. Given the significant role that this pathway may play in the uptake and assimilation of NO<sub>2</sub>, it is necessary that future research include multiple species comparisons of foliar uptake capacity, combined applications of multiple atmospheric N sources, and quantification of the fractionation events associated with foliar N uptake and how this influences foliar  $\delta^{15}\text{N}$  values. Results from such work will be imperative for the successful prediction of vegetative responses to increasing anthropogenic atmospheric N deposition.

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CHAPTER 3:  
INFLUENCE OF DIFFERENT ROOT NITROGEN SOURCES ON THE  
CAPACITY FOR DIRECT FOLIAR NO<sub>2</sub> UPTAKE AND ENZYME  
EXPRESSION IN *NICOTIANA TABACUM*

**Abstract**

Direct foliar uptake of gaseous reactive N has been observed in several plant species, but the mechanistic factors driving variation in foliar uptake capacity across species have yet to be rigorously tested. In this study, we investigated the influence of different root N sources (NO<sub>3</sub><sup>-</sup> vs. NH<sub>4</sub><sup>+</sup>) on enzyme expression and the capacity for foliar uptake of gaseous enriched <sup>15</sup>NO<sub>2</sub> in tobacco (*Nicotiana tabacum*) in a hydroponics-fumigation system. We measured differences in plant morphology, foliar δ<sup>15</sup>N, nitrate reductase (NR) activity, and glutamate synthetase (GS) activity to explore the potential drivers of foliar NO<sub>2</sub> uptake capacity. The amount of NO<sub>2</sub>-N incorporated into biomass via foliar uptake was not influenced by differences in the root N source. We observed altered enzyme activities and foliar C isotope ratios associated with NO<sub>2</sub> exposure and the form of N supplied to the roots. Specifically, leaf δ<sup>13</sup>C decreased, NR activity decreased, and GS activity increased in plants exposed to NO<sub>2</sub>, regardless of root N source. These results suggest that foliar uptake capacity is independent of the N form supplied to the roots and that enzyme expression within the primary N reduction pathways may be correlated with foliar uptake capacity in *N. tabacum*.

## Introduction

Reactive nitrogen (N) originating from human activity has dramatically increased in the past 50 years (Galloway *et al.* 2003, Vitousek 1994). A large proportion of this anthropogenic reactive N is emitted to the atmosphere and eventually deposited to terrestrial and marine ecosystems (Galloway *et al.* 2004). In particular, atmospheric levels of reactive N oxides ( $\text{NO}_y$ ) from combustion processes in vehicles and industry and ammonia gasses ( $\text{NH}_x$ ) from fertilizer and animal production have greatly increased the input of N to terrestrial ecosystems (Gessler *et al.* 2002, Morikawa *et al.* 1998).

Atmospheric reactive N addition to terrestrial ecosystems is potentially both an important source of nutrients to vegetation and a detriment to ecosystem function if N accumulates in excess of biological demand (Vitousek *et al.* 1997). In N-limited ecosystems, atmospheric reactive N deposition may represent a considerable source of N addition to vegetation and have a direct impact on plant metabolism (Hill 1971, Siegwolf *et al.* 2001, Sparks *et al.* 2001).

Although numerous studies have shown the ecological consequences of total N deposition on ecosystem processes (Aber *et al.* 2003, Galloway *et al.* 2003, e.g., Lovett 1994), few have partitioned and quantified the respective roles of different uptake pathways of N into vegetation. Two pathways of N addition that may play important roles in terrestrial ecosystems are root entry via bulk N deposition to the soil surface and the direct uptake of N into plant metabolism through the leaves. Although some plants meet their N demand exclusively by root uptake of ammonium ( $\text{NH}_4^+$ ), nitrate ( $\text{NO}_3^-$ ), and dissolved organic N from



the soil, many species are capable of incorporating atmospheric reactive N compounds, including  $\text{NO}_y$  and  $\text{NH}_x$ , directly through the leaves and assimilate this N into plant metabolism (Hereid & Monson 2001, Morikawa *et al.* 1998, Sparks *et al.* 2001, Sparks *et al.* 2003). Phytotoxicity resulting from direct foliar N uptake has been documented, although plants were exposed to unnaturally high levels of fumigation in these experiments (Zeevart 1976). In contrast, the chemical products of N assimilation via direct foliar uptake can be potential nutrients and are directly incorporated into metabolism to support growth at more realistic atmospheric reactive N concentrations (Gessler *et al.* 2000, Vallano & Sparks 2007). Since direct foliar N uptake may have a differential impact on plant metabolism compared to increased soil N availability and the subsequent root uptake of reactive N, then understanding the source N partitioning between these two pathways will be necessary for predicting future ecosystem function, structure and composition (Sparks 2009 and references therein).

Foliar uptake of nitrogen dioxide ( $\text{NO}_2$ ; a common atmospheric N pollutant) has been described in several studies, although few have explicitly tested the potential mechanistic factors driving the documented variation in foliar  $\text{NO}_2$  uptake capacity across species (Vallano & Sparks 2008 and references therein). Diffusion into the leaf, controlled by stomatal dynamics, appears to exert the strongest control on the rate of foliar N uptake and is likely the primary driver of variation in foliar N uptake capacity observed among plant taxa (Hanson & Lindberg 1991, Sparks *et al.* 2001, Weber & Rennenberg 1996). However, a wide variation in foliar uptake capacity still exists when stomatal conductance is constant across taxa (Morikawa *et al.* 1998). Thus, it

is likely that foliar  $\text{NO}_2$  uptake capacity is also influenced by a mesophyllic resistance (Gut *et al.* 2002, Teklemariam & Sparks 2006, Thoene *et al.* 1996), apoplastic antioxidant reactions by compounds such as ascorbate, and the subsequent removal of the primary end products of  $\text{NO}_2$  incorporation [nitrate ( $\text{NO}_3^-$ ) and nitrite ( $\text{NO}_2^-$ )] from the apoplast (Eller & Sparks 2006).

In this study, we specifically examined the influence of the rate of elimination of  $\text{NO}_3^-$  and  $\text{NO}_2^-$  from the apoplast in *Nicotiana tabacum*, a species that tends to express N reduction differentially in the plant body, with more than 80% of N reduction occurring in the leaves (Wakhloo & Staudt 1988). We investigated this potential control on foliar uptake capacity by providing the root system with different N sources ( $\text{NO}_3^-$  and  $\text{NH}_4^+$ ) and exposing the shoot systems to gaseous  $\text{NO}_2$ . Previous studies have reported that bean plants supplied with  $\text{NO}_3^-$  versus  $\text{NH}_4^+$  at the same concentration have higher rates of  $\text{NO}_2$  assimilation (Qiao & Murray 1997) and lower  $\text{NO}_2$  injury (Srivastava *et al.* 1992) in leaves. However, to better understand the complete range of mechanisms affecting the variation in foliar  $\text{NO}_2$  uptake capacity across plant species, it is necessary to examine the linkages between root N supply, associated assimilatory enzyme activity, and foliar  $\text{NO}_2$  uptake capacity.

Different root N sources may result in varying capacities for foliar  $\text{NO}_2$  uptake due to the nature of the associated assimilatory enzyme activities. Ammonium incorporated through the root uptake pathway is assimilated via glutamine synthetase (GS) primarily in the roots, although a small proportion of  $\text{NH}_4^+$  may also be transported to and assimilated in the leaves (Bloom 1988). In contrast,  $\text{NO}_3^-$  incorporated via root uptake can be reduced in either the root or

shoot tissue via nitrate reductase (NR) and eventually incorporated into amino acids and plant proteins. Gaseous reactive N compounds such as  $\text{NO}_2$  can be incorporated directly via foliar uptake and incorporated into N metabolism as either  $\text{NO}_3^-$  or  $\text{NO}_2^-$  ions, reduced to  $\text{NO}_2^-$  via NR activity if in the form of  $\text{NO}_3^-$ , converted from  $\text{NO}_2^-$  to  $\text{NH}_4^+$ , and eventually assimilated into organic compounds via the GS assimilatory enzyme pathway. Thus, the site and magnitude of activities of the NR and GS assimilatory enzyme pathways in response to both root and foliar uptake of various reactive N sources may also contribute to the large variation in foliar uptake capacity that has been documented across plant taxa (Eller & Sparks 2006, Gut *et al.* 2002, Hereid & Monson 2001, Morikawa *et al.* 1998, Teklemariam & Sparks 2004, Teklemariam & Sparks 2006, Thoene *et al.* 1996, Wellburn 1990).

The overarching goal of this study was to determine whether variation in root N sources and N reduction activity associated with different N assimilation pathways was directly correlated with foliar uptake capacity in *Nicotiana tabacum*. We measured the importance of different root N sources and their assimilation pathways on plant N metabolism using foliar N stable isotope ratios ( $^{15}\text{N}/^{14}\text{N}$ ) (Robinson 2001, Robinson *et al.* 1998), that often reflect the isotopic value of the N sources (Stewart *et al.* 2002). We used an established hydroponics-fumigation system in which plants were exposed to enriched gaseous  $^{15}\text{NO}_2$  as a tracer to create a separation in  $\delta^{15}\text{N}$  between N sources and allow for simultaneous and independent control of the concentrations and  $\delta^{15}\text{N}$  of multiple N sources (Vallano & Sparks 2007, Vallano & Sparks 2008). Using this system, we had the following explicit objectives: (1) to determine the influence of two different root N sources (e.g.  $\text{NH}_4^+$  versus  $\text{NO}_3^-$ ) on the

magnitude of foliar NO<sub>2</sub> uptake, the expression of enzyme activity, and plant performance, and (2) to examine the potential relationships among root N source, enzyme expression and the magnitude of foliar NO<sub>2</sub> uptake. We predicted that differential processing and assimilation of available root N sources would significantly impact the capacity for foliar NO<sub>2</sub> uptake in *N. tabacum*.

## **Materials and Methods**

### *Plants and treatments*

Tobacco (*N. tabacum*) seeds were purchased from a plant producer (Paramount Seeds, Inc., Palm City, FL, USA). Tobacco was selected for this study because it is a fast-growing herbaceous species that grows well in hydroponic systems. Seeds ( $n = 60$ ) were germinated and seedlings were grown in pots in perlite (Sun-Gro Horticulture, Bellevue, WA, USA) in a climate-controlled growth chamber (EGC, Chagrin Falls, OH, USA) at day and night temperatures of 27 and 21 °C, respectively, under moderate light (500  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) and a 16-h photoperiod. Plants were watered daily to saturation for two weeks and fertilized with a Hoagland solution including NO<sub>3</sub><sup>-</sup> or NH<sub>4</sub><sup>+</sup> of known  $\delta^{15}\text{N}$ . The  $\delta^{15}\text{N}$  values of the NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> nutrient solutions were  $-0.56 \pm 0.1\text{‰}$  and  $0.89 \pm 0.1\text{‰}$ , respectively. Forty-eight two-week-old plants were transplanted into the hydroponics-fumigation system and used for measurements. The remaining 12 plants were harvested and analyzed for biomass, morphology, and isotopic composition to provide a baseline for the experimental plants.

The hydroponics-fumigation system consisted of four 50 L opaque polyethylene nutrient tanks (120 × 58 × 16 cm) each fitted with airtight Plexiglas fumigation chambers (36 × 25 × 43 cm) with an opaque base. The system was located in a greenhouse with day and night temperatures of 27 and 21 °C, respectively, relative humidity of 60-70%, and moderate light conditions ( $800 \pm 75 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) using natural and supplemental metal halide lighting (400 MH Econo Cool Grow Light, Sunlight Supply, Vancouver, WA, USA) under a 16 h photoperiod. Plants were transplanted into the system with roots suspended in nutrient solution (20 °C) in a hydroponics tank and the shoots enclosed in a fumigation chamber maintained at ambient temperature. Each plant was stabilized and fitted with modeling clay (Loctite, Henkel Consumer Adhesives, Avon, OH, USA) at the root-shoot junction at the base of each chamber to ensure an impermeable seal between the fumigation and nutrient solution system components.

Two N regimes simulating low N supply (50 $\mu\text{M}$ ) were applied to the roots using nutrient solutions with fixed concentrations of either  $\text{NO}_3^-$  or  $\text{NH}_4^+$  as the sole root N source in a modified quarter-strength Hoagland solution (Smart & Bloom 1993). The  $\text{NO}_3^-$  and  $\text{NH}_4^+$  in the hydroponics nutrient solutions had the same  $\delta^{15}\text{N}$  values as the nutrient solution provided to plants prior to being placed in the system. The nutrient solutions were vigorously aerated at all times to provide adequate oxygenation and ensure complete mixing. Solution pH was maintained at 5.8-6.2 by the addition of either KOH or  $\text{H}_2\text{SO}_4$ . A 130 L reservoir of stock solution was used for each experimental N treatment. Nutrient solutions for each treatment were replaced weekly to minimize

microbial activity and prevent N depletion.  $\text{NO}_3^-$  and  $\text{NH}_4^+$  concentrations were measured weekly using an auto-analyzer (Astoria Pacific, Inc., Clackamas, OR, USA).

Fumigation chambers were supplied with filtered (activated charcoal), ambient air using a reciprocating air compressor (Model C403L, Gardner Denver, Quincy, IL, USA) at a flow rate of  $15 \text{ L min}^{-1}$ . Enriched  $^{15}\text{NO}_2$  from a compressed tank (1%  $\text{NO}_2$  : 99%  $\text{N}_2$ , Scott Marrin, Inc., Riverside, CA, USA) was diluted into the filtered air of selected chambers using high-precision rotometers and mass flow controllers (Models 03216-34 and 32044-00, Cole-Parmer, Vernon Hills, IL, USA) at a fixed partial pressure of 40 parts per billion (ppb). The  $\delta^{15}\text{N}$  of the enriched  $^{15}\text{NO}_2$  was  $1720\text{‰} \pm 17\text{‰}$ , providing a large signal separation from the  $\text{NO}_3^-$  and  $\text{NH}_4^+$  sources.  $\text{NO}_2$  was selected as the atmospheric N source because it is a common atmospheric reactive N compound. The fumigation treatment was selected to simulate urban (40 ppb) atmospheric  $\text{NO}_2$  concentrations. Six randomly selected chambers were used as controls and were not exposed to  $\text{NO}_2$  fumigation (0 ppb). Therefore, there were four treatments: (1) 0 ppb  $\text{NO}_2$  and  $50 \text{ }\mu\text{M NO}_3^-$ , (2) 40 ppb  $\text{NO}_2$  and  $50 \text{ }\mu\text{M NO}_3^-$ , (3) 0 ppb  $\text{NO}_2$  and  $50 \text{ }\mu\text{M NH}_4^+$ , and (4) 40 ppb  $\text{NO}_2$  and  $50 \text{ }\mu\text{M NH}_4^+$ . Within each treatment, there were three chambers with four plants/chamber ( $n = 12$ ).  $\text{NO}_2$  and nitric oxide (NO) concentrations were monitored using a chemiluminescence NO- $\text{NO}_2$ - $\text{NO}_x$  analyzer (TECO Model 42, Thermo Environmental Instruments, Inc., Franklin, MA, USA). Non-incorporated  $\text{NO}_2$  was filtered using activated charcoal and exited the system through an output line extending outside the greenhouse. Temperature and relative humidity within the chambers were monitored using humidity and

temperature probes (Model HMP45A, Vaisala, Inc., Boulder, Colorado, USA) connected to a datalogger (Model CR10x, Campbell Scientific, Inc., Logan, Utah, USA).

### *Morphological and Stable Isotope Analysis*

Plants were harvested 28 days after the start of fumigation (or at the beginning of the experiment for baseline plants). Whole-plant samples were separated into leaf, shoot, and root tissue. Shoot length and leaf number were measured for each individual and leaf area was estimated using a leaf area meter (LI-3100 Area Meter, LI-COR, Inc., Lincoln, NE, USA). Plant tissue samples were dried, weighed, and sub-samples analyzed for tissue N and C content and  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$ . Previously separated tissues samples were rinsed with deionized water to remove any  $\text{NO}_2$  deposited to the leaf surface and dried for three days at 55 °C in a drying oven. The dried tissue samples were weighed using a balance (Model M-2200, Denver Instrument, Denver, CO, USA), ground to a fine powder with a mortar and pestle, and sub-samples of 2.55-3.15 mg were weighed for isotopic analysis using a microbalance (Model 4504MP8; Sartorius Corp. Edgewood, NY, USA). Tissue  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  were measured using a continuous flow isotope ratio mass spectrometer (Model Delta Plus; Thermo Finnigan, San Jose, CA, USA). Tissue N and C contents were measured using a CHN elemental analyzer (Model Carlo Erba NC2500; Thermo Finnigan, San Jose, CA, USA). All analyses were conducted at the Stable Isotope Laboratory at Cornell University.

## *Enzyme Assays*

Samples were weighed and immediately stored in liquid N until the preparation of the extracts. Root and leaf NR activity was assayed *in vitro* from 12 plants per treatment according to Scheible *et al.* (1997). NR activity was determined as the rate of  $\text{NO}_2^-$  accumulation and was measured at 540 nm using a spectrophotometer (Beckman DU 640). The samples were compared with a standard curve generated using a  $\text{KNO}_2$  stock solution. Root and leaf GS activity was assayed *in vitro* from 12 plants per treatment according to Hazelton (2007). GS activity was determined as the rate of gammaglutamylhydroxamic acid (GHA) accumulation and was measured at 540 nm using a spectrophotometer (Beckman DU 640). The samples were compared with a standard curve generated using a GHA stock solution.

## *Calculation of N Source Partitioning*

Partitioning of plant N among different uptake pathways (foliar uptake of gaseous  $\text{NO}_2$  and root uptake of nutrient solution  $\text{NO}_3^-$  or  $\text{NH}_4^+$ ) was calculated using a two ended linear mixing model (Dawson *et al.* 2002) and published fractionation factors for root  $\text{NH}_4^+/\text{NO}_3^-$  assimilation (Kolb & Evans 2003). Because we used an artificially high enrichment of  $^{15}\text{N}$  in the  $\text{NO}_2$ , fractionation events associated with foliar uptake were likely not detectable (i.e., the signal separation generated by the tracer was much larger than all natural fractionations). Using this model, we defined the total amount of  $\text{NO}_2$  incorporated via direct foliar uptake during the fumigation period.



## Statistical Analysis

Data were statistically evaluated using a multifactorial analysis of variance (ANOVA), with N in the nutrient solution ( $\text{NO}_3^-$  or  $\text{NH}_4^+$ ) and  $\text{NO}_2$  fumigation treatments as the two factors. We used the Tukey's HSD procedure to make pair wise comparisons. Significant relationships between two variables were determined by simple linear regressions. Statistical analyses were performed with SAS JMP software (Version 7.0, 2007) and using a significance level of  $\alpha = 0.05$ .

## Results

### *Foliar Uptake and Nitrogen Source Partitioning*

We found no significant differences in the total proportion and amount of N incorporated via foliar  $\text{NO}_2$  uptake into total biomass between plants grown under  $\text{NO}_3^-$  versus  $\text{NH}_4^+$  root supply (Fig. 3.1). When exposed to gaseous  $\text{NO}_2$ , plants grown on  $\text{NO}_3^-$  or  $\text{NH}_4^+$  incorporated  $11.6 \pm 1.4$  and  $11.5 \pm 1.4$  mg  $\text{NO}_2\text{-N}$ , respectively, into total biomass via foliar  $\text{NO}_2$  uptake ( $P < 0.0001$ ). This amount represents  $12.0 \pm 0.6$  and  $12.4 \pm 0.6\%$  of total plant N in fumigated plants grown on  $\text{NO}_3^-$  or  $\text{NH}_4^+$ , respectively. Regardless of root N supply, the amount of total  $\text{NO}_2\text{-N}$  incorporated via foliar  $\text{NO}_2$  uptake was significantly higher in leaf tissue compared to root and shoot tissues. In fumigated individuals, 88% and 87% of  $\text{NO}_2\text{-N}$  from foliar  $\text{NO}_2$  uptake was assimilated into leaf tissue compared to root and shoot tissues in plants grown under root  $\text{NO}_3^-$  and  $\text{NH}_4^+$ , respectively. Similar to the total plant measurements, the total

amount of  $\text{NO}_2\text{-N}$  incorporated into specific tissue types was not influenced by root N supply. Foliar  $\delta^{15}\text{N}$  values for fumigated plants grown under the  $\text{NO}_3^-$  and  $\text{NH}_4^+$  treatments were  $206.1 \pm 10.2\text{‰}$  and  $215.9 \pm 10.6\text{‰}$ , respectively.

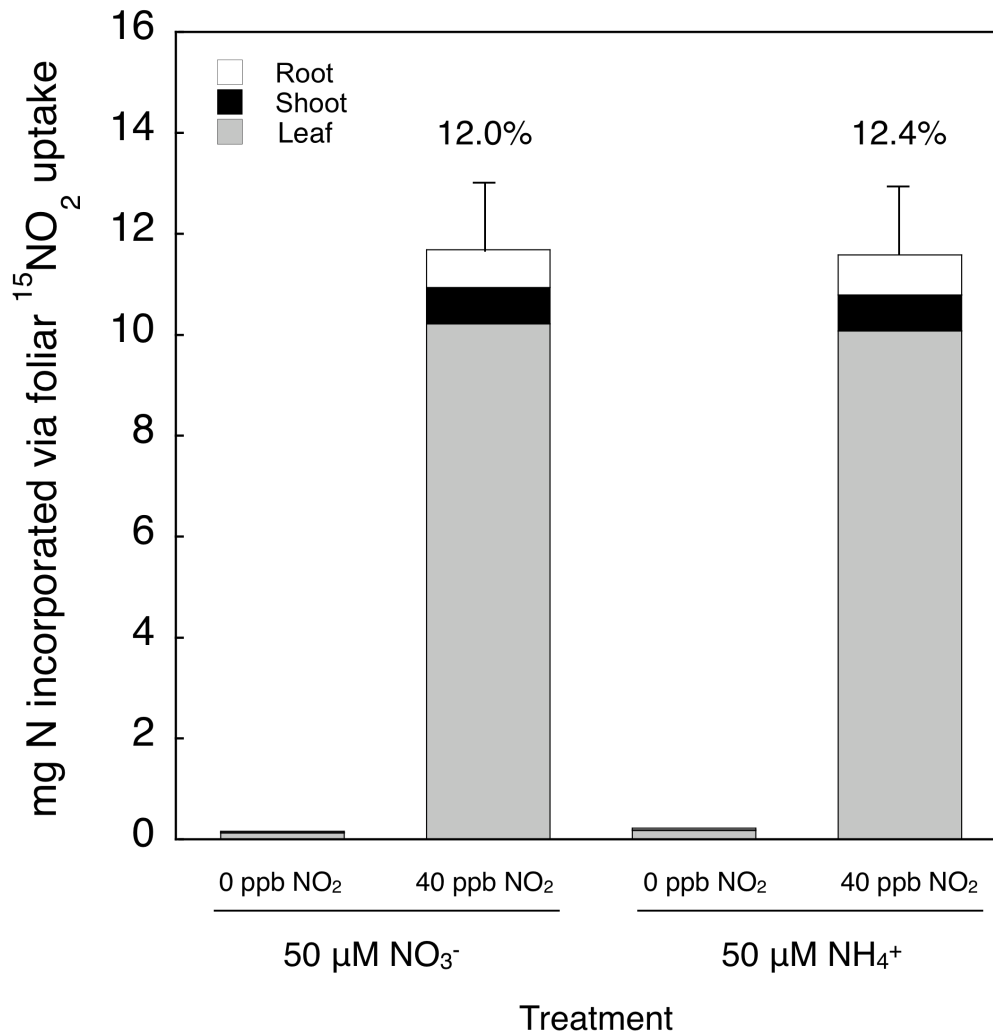


Figure 3.1. Amount (in mg) of total nitrogen content attributable to the direct foliar uptake of  $^{15}\text{NO}_2\text{-N}$  in *N. tabacum* and partitioning of N by tissue type for roots (white), shoots (black), and leaves (grey). Proportion (%) of total nitrogen content attributable to the direct foliar uptake of  $^{15}\text{NO}_2\text{-N}$  is highlighted above bars. Data are means  $\pm$  1 standard error ( $n = 12$ ). Different lower-case letters indicate statistically significant differences ( $P \leq 0.05$ ) across treatments.

### *Morphology and biomass allocation*

*N. tabacum* did not show changes in total biomass accumulation in response to different root N sources (Fig. 3.2a). Further, plants fumigated with gaseous  $\text{NO}_2$  showed similar total biomass accumulation compared to non-fumigated individuals, regardless of the root N source. We observed a non-significant trend for greater total biomass accumulation in plants exposed to  $\text{NO}_2$  and grown under  $\text{NO}_3^-$  supply (by 29%,  $P = 0.16$ ), but this weak pattern was not observed in plants grown on  $\text{NH}_4^+$  supply. We did not find any differences in the ratio of root to shoot (stem + leaf) tissue in non-fumigated plants grown on  $\text{NO}_3^-$  versus  $\text{NH}_4^+$  (Fig. 3.2b). However,  $\text{NO}_2$  exposure did stimulate shoot growth relative to root growth in plants grown under  $\text{NO}_3^-$ , causing a decrease (by 22%) in the root to shoot ratio ( $P = 0.003$ ). We did not observe this pattern for fumigated plants grown under  $\text{NH}_4^+$ .

Differences in C and N allocation patterns were observed in response to both root N source and  $\text{NO}_2$  exposure (Fig. 3.3a). The C:N ratio was lower in non-fumigated plants grown under  $\text{NH}_4^+$  versus  $\text{NO}_3^-$  ( $P = 0.047$ ). However, this trend reversed under  $\text{NO}_2$  fumigation with the C:N ratio being higher in fumigated plants grown under  $\text{NH}_4^+$  versus  $\text{NO}_3^-$  ( $P = 0.011$ ). Fumigated plants exhibited higher a C:N ratio than non-fumigated plants grown under root  $\text{NH}_4^+$  ( $P < 0.0001$ ). However, there were no differences in the C:N ratio between fumigated and non-fumigated plants grown under  $\text{NO}_3^-$ . Overall, leaf  $\delta^{13}\text{C}$  values were higher in plants grown under  $\text{NH}_4^+$  versus  $\text{NO}_3^-$ , regardless of  $\text{NO}_2$  exposure ( $P < 0.01$ , Fig. 3.3b). Further, leaf  $\delta^{13}\text{C}$  values were more negative in

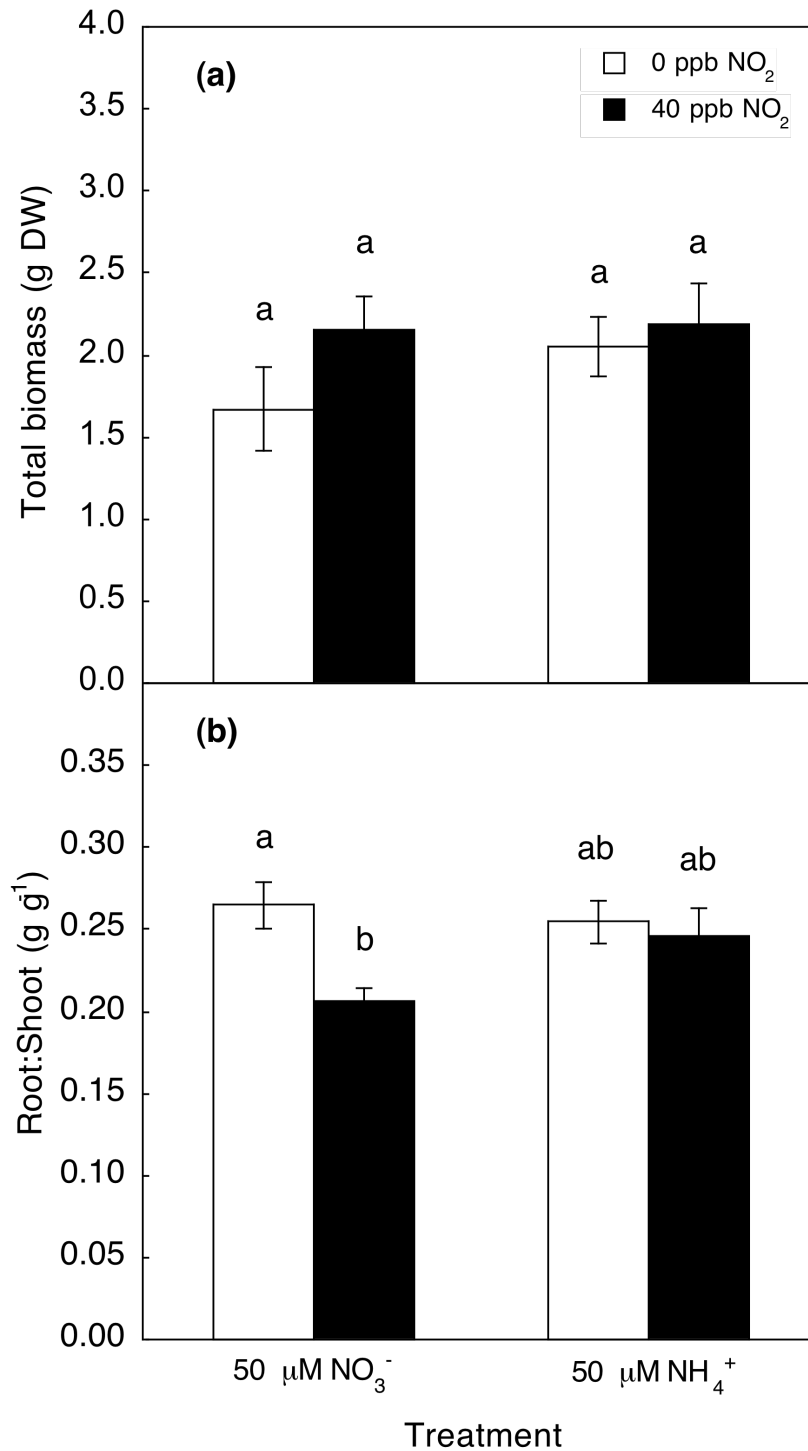


Figure 3.2. Total plant biomass (a) and root to shoot ratio (b) in *N. tabacum*. Open bars represent plants exposed to  $0 \text{ ppb}$  gaseous  $^{15}\text{NO}_2$  and closed bars represent plants exposed to  $40 \text{ ppb}$  gaseous  $^{15}\text{NO}_2$ . Data are means  $\pm 1 \text{ SE}$  ( $n = 12$ ). Different lower-case letters indicate statistically significant differences ( $P \leq 0.05$ ) across treatments.

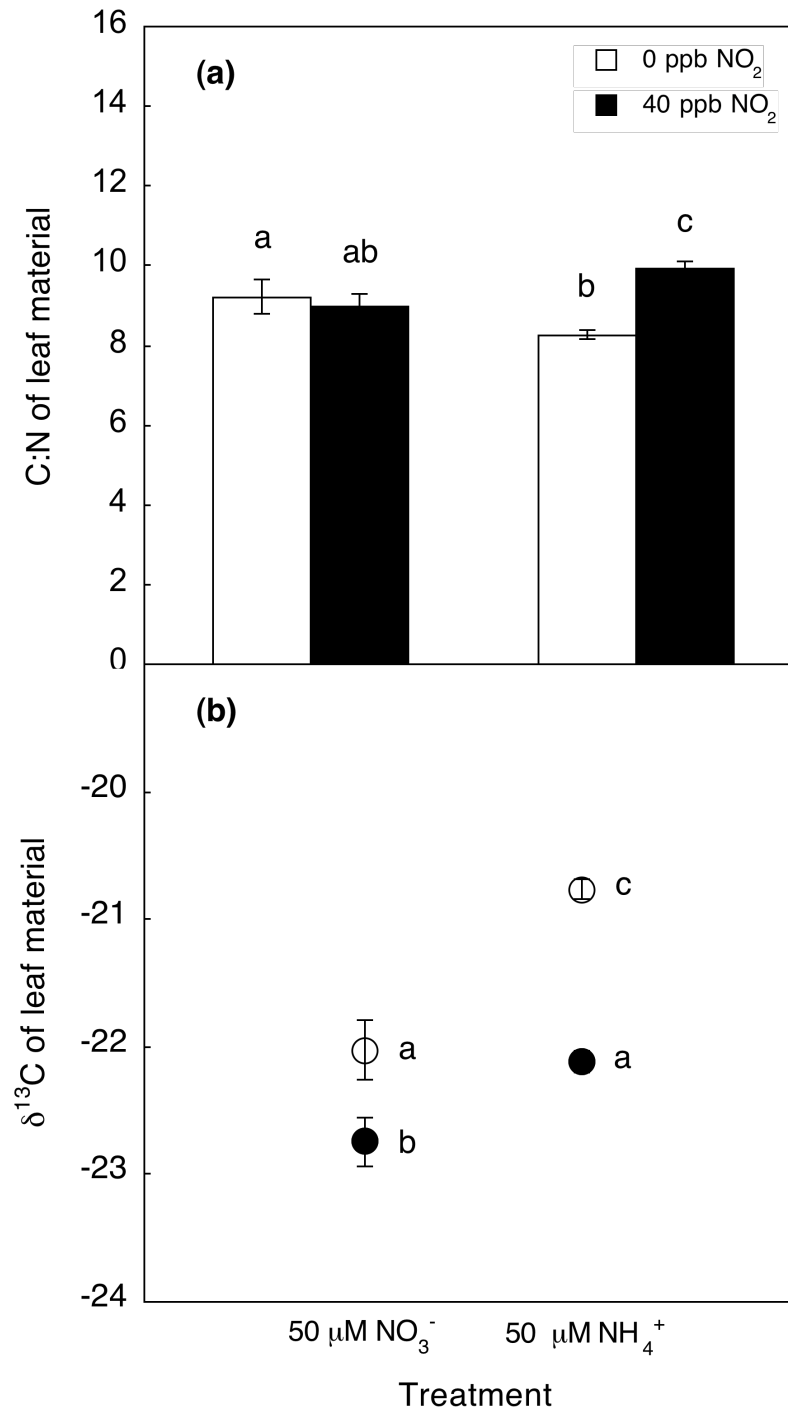


Figure 3.3. Carbon to nitrogen ratio (a) and foliar  $\delta^{13}\text{C}$  (b) in *N. tabacum*. Open bars and circles represent plants exposed to 0 ppb gaseous  $^{15}\text{NO}_2$  and closed bars and circles represent plants exposed to 40 ppb gaseous  $^{15}\text{NO}_2$ . Data are means  $\pm$  1 SE ( $n = 12$ ). Different lower-case letters indicate statistically significant differences ( $P \leq 0.05$ ) across treatments.

fumigated plants grown under  $\text{NO}_3^-$  ( $P < 0.0001$ ) and  $\text{NH}_4^+$  ( $P = 0.027$ ) when compared to non-fumigated plants.

### *Enzyme Activity*

We found no differences in root NR activity between non-fumigated plants grown on  $\text{NH}_4^+$  versus  $\text{NO}_3^-$  (Fig. 3.4a). However, root NR activity was higher in fumigated plants grown on  $\text{NO}_3^-$  compared to  $\text{NH}_4^+$  ( $P = 0.002$ ). Within either root N source treatment, there were no differences in root NR activity between fumigated and non-fumigated plants. Leaf NR activity was higher in plants grown on  $\text{NO}_3^-$  compared to plants grown on  $\text{NH}_4^+$ , regardless of  $\text{NO}_2$  exposure ( $P < 0.05$ , Fig. 3.4b). However, leaf NR activity decreased in fumigated plants grown under  $\text{NO}_3^-$  ( $P = 0.024$ ) or  $\text{NH}_4^+$  ( $P = 0.0002$ ).

Root GS activity was higher in plants grown on  $\text{NH}_4^+$  versus  $\text{NO}_3^-$ , regardless of  $\text{NO}_2$  exposure ( $P < 0.0001$ , Fig 3.5a). We found no changes in root GS activity in fumigated plants grown on  $\text{NO}_3^-$ , while root GS activity increased in fumigated plants grown on  $\text{NH}_4^+$  supply ( $P = 0.0264$ ). Overall, leaf GS activity was higher in plants grown on  $\text{NH}_4^+$  compared to plants grown on  $\text{NO}_3^-$ , regardless of  $\text{NO}_2$  exposure ( $P < 0.05$ , Fig. 3.5b). Leaf GS activity increased in fumigated plants grown on  $\text{NO}_3^-$  ( $P = 0.0078$ ) and  $\text{NH}_4^+$  ( $P < 0.0001$ ).

When we examined the entire range of leaf NR activity expressed under  $\text{NO}_2$  fumigation, we found a significant correlation between leaf NR activity and the amount of  $\text{NO}_2$ -N incorporated via foliar uptake in plants grown on  $\text{NH}_4^+$  ( $P = 0.04$ ,  $r^2 = 0.52$ ), but no significant relationship was observed between these

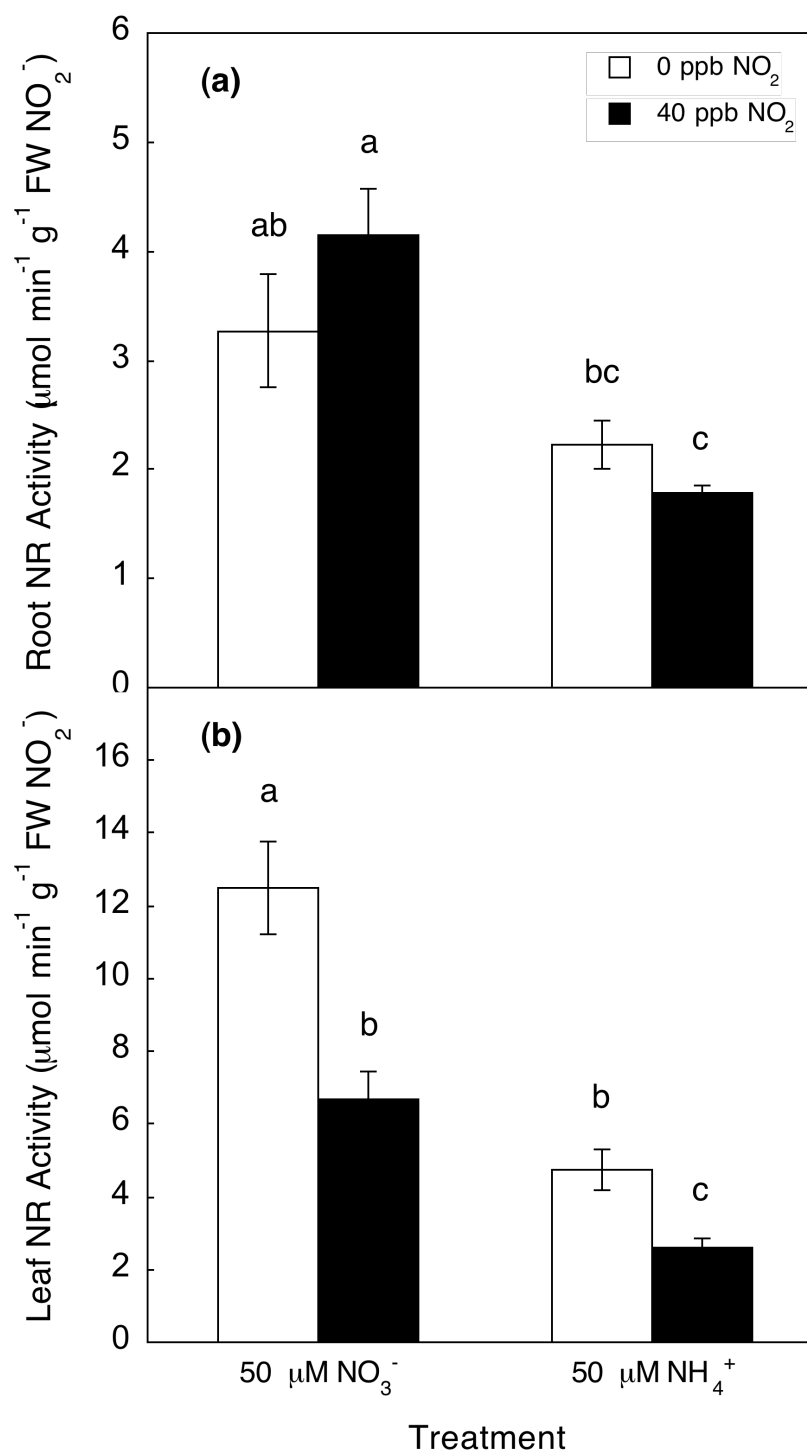


Figure 3.4. Root (a) and leaf nitrate reductase (NR) activity (b) in *N. tabacum*. Open bars represent plants exposed to 0 ppb gaseous  $^{15}\text{NO}_2$  and closed bars represent plants exposed to 40 ppb gaseous  $^{15}\text{NO}_2$ . Data are means  $\pm 1$  SE ( $n = 12$ ). Different lower-case letters indicate statistically significant differences ( $P \leq 0.05$ ) across treatments.

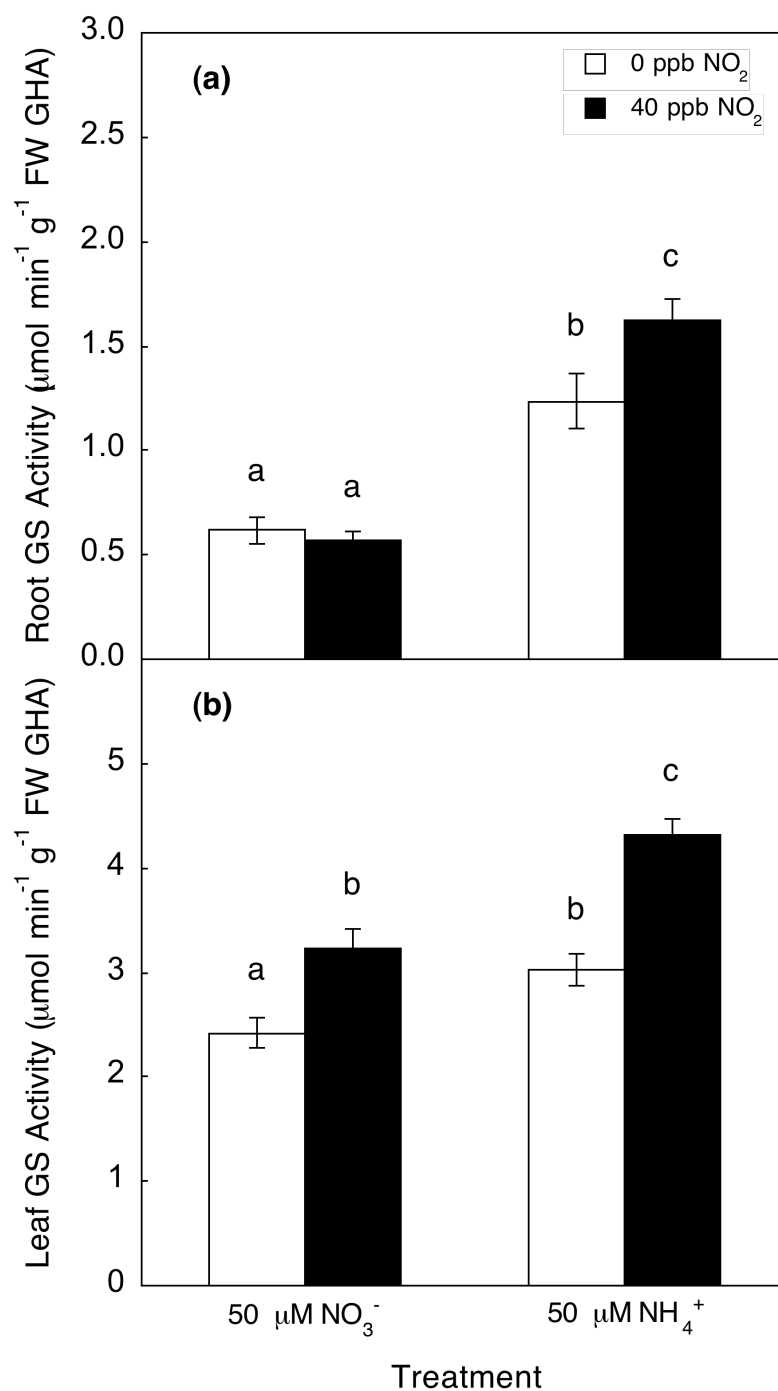


Figure 3.5. Root (a) and leaf glutamine synthetase (GS) activity (b) in *N. tabacum*. Open bars represent plants exposed to 0 ppb gaseous  $^{15}\text{NO}_2$  and closed bars represent plants exposed to 40 ppb gaseous  $^{15}\text{NO}_2$ . Data are means  $\pm$  1 SE ( $n = 12$ ). Different lower-case letters indicate statistically significant differences ( $P \leq 0.05$ ) across treatments.



factors in plants grown under  $\text{NO}_3^-$  (Fig. 3.6a). Overall, leaf GS activity was not correlated with the amount of  $\text{NO}_2\text{-N}$  incorporated via foliar uptake, regardless of the root N source (Fig. 3.6b).

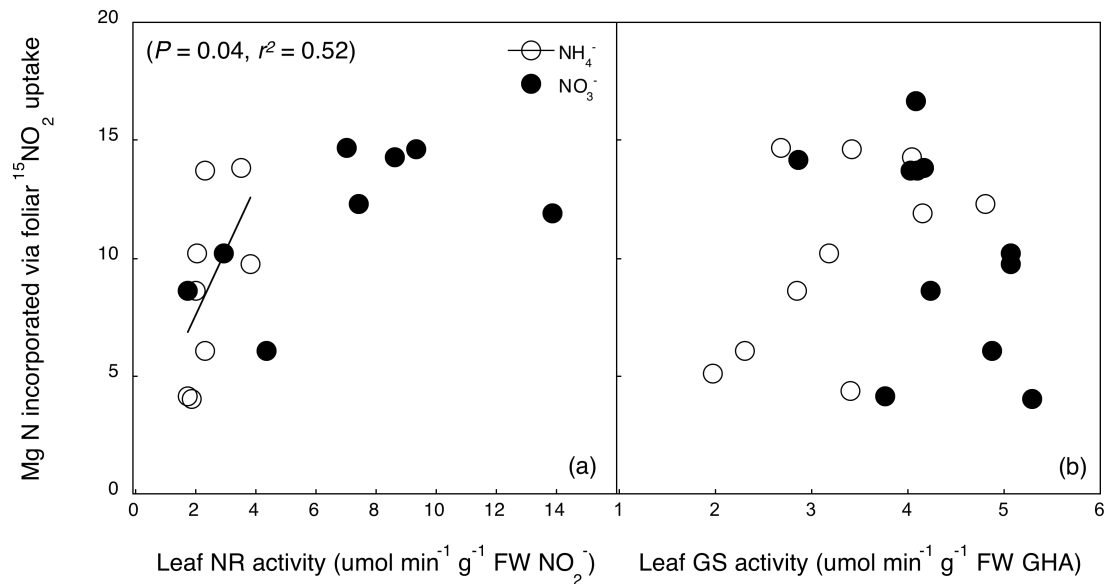


Figure 3.6. Relationship between leaf NR activity vs. mg N incorporated via foliar  $^{15}\text{NO}_2$  uptake (a) and leaf GS activity and mg N incorporated via foliar  $^{15}\text{NO}_2$  uptake (b) in *N. tabacum* ( $n = 12$ ). Plants were supplied with either  $\text{NO}_3^-$  (closed circles and dashed lines) or  $\text{NH}_4^+$  (open circles and solid lines) as their sole root N source.

## Discussion

The existence of the foliar N uptake pathway and some potential factors influencing foliar uptake capacity across taxa has been documented in the literature (Gessler *et al.* 1998, Hereid & Monson 2001, Hill 1971, Muller *et al.* 1996, Sparks *et al.* 2001, Takahashi *et al.* 2005). This study extends previous work by differentiating between deposited N among separate uptake pathways (soil vs. foliar) and investigating the influence of different root N sources and changes in downstream enzyme activity on foliar uptake capacity. We show

that the proportion and amount of NO<sub>2</sub>-N incorporated into biomass via direct foliar uptake was not influenced by differences in the root N source (Fig. 3.1), suggesting that the overall rate of foliar NO<sub>2</sub> uptake is not strongly impacted by the form of N supplied to the root system in *N. tabacum*. This was the case in spite of the observation of altered enzyme activities associated with availability of different root N sources and NO<sub>2</sub> exposure (Figs. 3.4, 3.5, 3.6).

Our results suggest that the rate of foliar NO<sub>2</sub> uptake is limited by resistances other than the activity of downstream enzymes in the N reduction pathway. Several studies have reported that the rate of foliar NO<sub>2</sub> uptake is highly dependent on stomatal conductance (Eller & Sparks 2006, Gessler *et al.* 2000, Hanson & Lindberg 1991, Thoene *et al.* 1991, Weber & Rennenberg 1996). Further, N availability may influence stomatal conductance, photosynthetic rate, or both simultaneously, subsequently influencing foliar  $\delta^{13}\text{C}$  (Farquhar *et al.* 1982, Schulze *et al.* 1996). We observed lower foliar  $\delta^{13}\text{C}$  in response to NO<sub>2</sub> exposure regardless of the root N source (Fig 3.3b), which is indicative of an increase in the intercellular CO<sub>2</sub> concentration ( $c_i$ ) compared to the ambient CO<sub>2</sub> concentration ( $c_a$ ). This pattern may be due to an increase in photosynthetic capacity and stomatal conductance in response to increased N supply via the foliar NO<sub>2</sub> uptake pathway. However, we observed no differences in leaf N content in response to NO<sub>2</sub> exposure regardless of root N source. Therefore, it is likely that the decrease in foliar  $\delta^{13}\text{C}$  primarily reflects changes in stomatal conductance due to direct foliar NO<sub>2</sub> uptake in fumigated plants.

Additionally, mesophyllic resistances likely play an important role in regulating rates of foliar  $\text{NO}_2$  uptake (Rondon *et al.* 1993, Sparks *et al.* 2001, Thoene *et al.* 1991, Weber & Rennenberg 1996), although these resistances have not been conclusively identified. Internal resistance to foliar  $\text{NO}_2$  uptake has been documented in some studies (Thoene *et al.* 1991), but not in others (Neubert *et al.* 1993). For instance, internal diffusion of gaseous  $\text{NO}_2$  into the leaf during the liquid phase versus the gaseous phase may be limited by the internal surface area of the chloroplasts exposed to intercellular air spaces per unit leaf area (Evans 1999, Evans *et al.* 1994). Other internal resistances include the disproportionation reaction of  $\text{NO}_2$  to  $\text{NO}_2^-$  and  $\text{NO}_3^-$  ions in the leaf apoplast by free-radical scavenging compounds such as ascorbate (Sparks *et al.* 2001) and differential permeability of  $\text{NO}_3^-/\text{NO}_2^-$  ions through cell membranes and walls (Ammann *et al.* 1995). Any one of these factors or, more likely, a combination of them likely drives much of the species-level variation observed in plant foliar uptake of  $\text{NO}_2$  at constant leaf conductance (e.g. Morikawa *et al.* 1998).

Root N supply and  $\text{NO}_2$  exposure did not appear to have a strong influence on plant growth, although we observed some changes nutrient allocation (Fig. 3.2). For instance, fumigated plants grown on  $\text{NO}_3^-$  supply had decreased ratios of belowground to aboveground tissue. This observation, as noted by others (Nussbaum *et al.* 1999, Siegwolf *et al.* 2001), suggests that  $\text{NO}_2$  fumigation directly or indirectly induces changes in basic plant allocation patterns. Such a change could have indirect consequences for growth over the long term such as plant tolerance to drought, low nutrient conditions, or other forms of environmental stress.

We observed changes in both root and leaf enzyme expression in response to different root N source treatments (Figs. 3.4, 3.5). It is well known that  $\text{NO}_3^-$  reduction requires the activity of NR, which is a substrate-inducible enzyme (Matt *et al.* 2001). Higher root and leaf NR activities in plants grown on  $\text{NO}_3^-$  versus  $\text{NH}_4^+$  root supply were likely a consequence of a greater  $\text{NO}_3^-$  substrate pool in each plant organ due to  $\text{NO}_3^-$  being the primary root N source. In contrast, it has been documented that root N form has little or no effect on GS activity (Claussen & Lenz 1999) or may show an increase in GS activity in the presences of  $\text{NH}_4^+$  (Cruz *et al.* 2006, Lasa *et al.* 2002). We found higher root GS activity in plants grown on  $\text{NH}_4^+$  versus  $\text{NO}_3^-$ . One possible mechanism driving this observation would be supplying roots with only  $\text{NH}_4^+$  leads to a large  $\text{NH}_4^+$  substrate pool in the roots and necessitates high GS activity to avoid toxic accumulation of  $\text{NH}_4^+$ . However, we also observed higher leaf GS activity in plants grown on  $\text{NH}_4^+$  versus  $\text{NO}_3^-$ , a result reported by other studies on orchids (Majerowicz *et al.* 2000) and soybean and pea (Forde & Clarkson 1999).

Leaf NR and GS enzyme activities differed in their responses to  $\text{NO}_2$  exposure, regardless of the root N source. Previous work has reported that  $\text{NO}_2$  exposure stimulates NR activity (Eller & Sparks 2006, Murray & Wellburn 1985, Rowland *et al.* 1989), although inhibition of foliar N uptake has also been documented (Qiao & Murray 1998 and references therein). We observed a decrease in leaf NR activity in response to  $\text{NO}_2$  exposure, indicating that  $\text{NO}_2$  exposure may be limiting NR activity and hence,  $\text{NO}_3^-$  assimilation in *N. tabacum* (Fig. 3.4b). In contrast, we observed a positive relationship between leaf GS activity and the amount of  $\text{NO}_2$  incorporated via foliar uptake

regardless of root N supply (Fig. 3.5b). These results suggest the potential of leaf GS activity stimulation in response to an increase in the overall flux of N at the leaf level from NO<sub>2</sub> exposure.

When the entire range of foliar NO<sub>2</sub> uptake was considered, we found a positive correlation between leaf NR activity and the amount of NO<sub>2</sub> incorporated via foliar uptake in plants grown under NH<sub>4</sub><sup>+</sup>, suggesting the potential for leaf NR activity to stimulate foliar NO<sub>2</sub> uptake under these conditions. However, there was no significant correlation between these factors in plants grown under NO<sub>3</sub><sup>-</sup> (Fig. 3.6a). We did not find a significant relationship between leaf GS activity and the amount of NO<sub>2</sub> incorporated via foliar uptake regardless of the root N source (Fig. 3.6b).

The decrease in leaf NR activity and increase in GS activity under NO<sub>2</sub> exposure may be due to several factors. Preferential disproportionation of NO<sub>2</sub> into NO<sub>2</sub><sup>-</sup> rather than NO<sub>3</sub><sup>-</sup> in the leaf apoplast based on the activity of ascorbate and other antioxidant compounds is one potential scenario that may be driving the decrease in NR activity under NO<sub>2</sub> exposure (Eller & Sparks 2006, Ramge *et al.* 1993). Under these circumstances, NO<sub>2</sub> entering the leaf apoplast via foliar uptake would react preferentially with ascorbate rather than water to yield more NO<sub>2</sub><sup>-</sup> versus NO<sub>3</sub><sup>-</sup>, respectively (Gessler *et al.* 1998, Sparks 2009). This proportionation reaction is irreversible and dependent on the apoplastic concentration of NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> ions and the presence of apoplastic antioxidants such as ascorbate that may scavenge these ions (Stulen *et al.* 1998). We suggest that if NO<sub>2</sub><sup>-</sup> were the primary chemical product of gaseous NO<sub>2</sub> uptake and dissolution in the apoplast, then this

compound would enter the  $\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NH}_4^+$  pathway further downstream than  $\text{NO}_3^-$ , resulting in decreased NR activity and increased GS activity compared to non-fumigated plants.

Although we did not observe any detrimental effects of  $\text{NO}_2$  exposure on plant growth, another plausible mechanism for the observed patterns in enzyme activity is toxicity from  $\text{NO}_2$  exposure. Previous work has acknowledged the potential phytotoxic effects of  $\text{NO}_2$  exposure on plant metabolism at unrealistically high ambient concentrations of 4-11 parts per million (ppm), although these effects have yet to be documented at realistic concentrations (Wellburn 1990, Zeevart 1976). Specifically, the dissolution of  $\text{NO}_2$ -N in the apoplast originating from foliar uptake may yield a net  $\text{H}^+$  production and increases in  $\text{NO}_3^-$  concentration, that can then exceed the proton buffering capacity in the apoplast and result in toxicity (Rennenberg & Gessler 1999, Stulen *et al.* 1998), ultimately leading to the inhibition of NR activity observed in our study. This scenario is consistent with Qiao and others (1997, 1998), who have also noted declines in the rate of foliar  $\text{NO}_2$  uptake likely due to accumulation of  $\text{H}^+$  in the leaf apoplast from  $\text{NO}_2$  exposure in plants grown on either root  $\text{NO}_3^-$  or  $\text{NH}_4^+$  supply.

Feedback inhibition between the NR and the GS reduction pathways may also contribute to the observed patterns in enzyme activity. Most likely,  $\text{NO}_3^-$  uptake and the  $\text{NO}_3^-$  and  $\text{NH}_4^+$  assimilatory pathways are closely interlinked processes (Matt *et al.* 2001). For instance, NR activity may have been influenced not only by  $\text{NO}_2$  exposure, but also by the presence of  $\text{NH}_4^+$  incorporated via the roots and translocated to the leaves. The increase in leaf

GS activity in response to  $\text{NO}_2$  exposure observed in this study (Fig. 3.4b) suggests that foliar  $\text{NO}_2$  uptake may increase GS activity either by inducing its synthesis or activity or by preventing degradation or inactivation of the enzyme. Enhancement of this step of the N assimilatory pathway may be directly linked to the decrease in leaf NR activity under  $\text{NO}_2$  exposure. NR activity is often suppressed in the presence of  $\text{NH}_4^+$  (Srivastava & Ormrod 1984). Specifically,  $\text{NH}_4^+$  can inhibit  $\text{NO}_3^-$  uptake, inactivate the NR system or stop or slow the synthesis of active NR enzyme (Gessler *et al.* 1998).

The results of this study suggest that plant performance and foliar uptake capacity are independent of the root N form and that enzyme expression within the primary N assimilatory pathways may be correlated with foliar uptake capacity in *N. tabacum*. However, the observed changes in enzyme activity associated with N reduction indicate that the effects of foliar  $\text{NO}_2$  uptake at the physiological level may have important long-term impacts on plant metabolism. It is clear that foliar  $\text{NO}_2$  uptake contributes a significant amount of N to plants at the whole-plant level, although the effects of this uptake pathway on enzymatic processes have yet to be conclusively determined. Further work focused on the comparisons of foliar uptake capacity across multiple species, potential mesophyllic resistances to foliar uptake, and quantification of the within-plant N fractionation events associated with foliar N uptake will be crucial for predicting how vegetation will respond to increasing atmospheric N deposition.

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CHAPTER 4:  
PARTITIONING THE RELATIVE INFLUENCE OF SOIL N, MYCORRHIZAE,  
AND DIRECT FOLIAR N UPTAKE ON FOLIAR  $\delta^{15}\text{N}$  PATTERNS: CAN WE  
DETECT FOLIAR UPTAKE OF REACTIVE N?

**Abstract**

Vegetation is an important sink for atmospheric reactive N in N-limited systems and may be capable of incorporating reactive N compounds directly into leaves through the foliar uptake pathway. A proxy for atmospheric reactive N entering vegetation would be useful to estimate the impact of direct foliar N uptake on plant metabolism. Natural abundance foliar N isotopic composition ( $\delta^{15}\text{N}$ ) is a practical tool for this purpose because plant-available N sources often have different isotopic compositions. Current understanding of foliar  $\delta^{15}\text{N}$  suggests these values primarily represent the integration of soil  $\delta^{15}\text{N}$ , direct foliar N uptake, mycorrhizal fractionation, and within-plant fractionations. Using a potted plant mesocosm system, we estimated the influence of mycorrhizae on foliar  $\delta^{15}\text{N}$  patterns in red maple (*Acer rubrum*) seedlings along an N deposition gradient in New York State. We found that mycorrhizal associations altered foliar  $\delta^{15}\text{N}$  in red maple seedlings from 0.03 – 1.01‰ across sites. Along the same temporal and spatial scales, we examined the influence of soil  $\delta^{15}\text{N}$ , foliar N uptake, and mycorrhizae on foliar  $\delta^{15}\text{N}$  in adult stands of American beech (*Fagus grandifolia*), black birch (*Betula lenta*), red maple (*A. rubrum*), and red oak (*Quercus rubra*). Using multiple regression models, atmospheric  $\text{NO}_2$  concentration explained 0%, 69%, 23%, and 45% of the residual variation in foliar  $\delta^{15}\text{N}$  remaining in American beech, red maple, red

oak, and black birch, respectively, after accounting for soil  $\delta^{15}\text{N}$ . Our results suggest that foliar  $\delta^{15}\text{N}$  may be used to estimate pollution-derived atmospheric reactive N entering vegetation via the foliar N uptake pathway.

## Introduction

Over the last century, human activity has greatly increased the amount of biologically reactive nitrogen (N) entering the natural environment and this trend is predicted to continue over the coming decades. A large proportion of reactive N is transported to the atmosphere and eventually deposited to terrestrial and marine ecosystems. On a global scale, atmospheric reactive N deposition has increased from 31.6 to 103 Tg N yr<sup>-1</sup> from 1860 to the mid-1990s and is projected to increase to 195 Tg N yr<sup>-1</sup> by 2050 (Galloway *et al.* 2003, Galloway *et al.* 2004). In particular, the rise in atmospheric levels of N oxides (NO<sub>y</sub>) and ammonia gases (NH<sub>x</sub>) originating from fertilizer use and fossil fuel combustion has significantly increased the input of wet and dry N deposition to terrestrial ecosystems. This drastic increase in atmospheric reactive N levels and the resulting N that is subsequently deposited to plants and soil is often associated with dramatic changes in the structure, function and composition of ecosystems (Vitousek *et al.* 1997).

The short and long-term ecological consequences of increased atmospheric reactive N deposition can be potentially beneficial to vegetation by increasing the availability of nutrient sources and detrimental to ecosystem function if N accumulates in excess of biological demand (Driscoll *et al.* 2003, Vitousek *et al.* 1997). For instance, increased atmospheric reactive N deposition has been

associated with detrimental ecological effects, such as soil acidification, plant nutrient imbalances leading to forest decline, and changes in species composition (Aber 1992, Schaberg *et al.* 2002). In N-limited ecosystems, such as the temperate forests in the northeastern US, atmospheric reactive N deposition may represent a considerable source of N addition to plants and may have beneficial implications for plant metabolism (Hill 1971, Siegwolf *et al.* 2001, Sparks *et al.* 2001). Although numerous studies have shown the ecological consequences of total N deposition on ecosystem processes (Aber *et al.* 2003, Galloway *et al.* 2003, e.g., Lovett 1994), few have partitioned and quantified the respective roles of the primary uptake pathways of atmospheric reactive N into vegetation.

The pathways of N addition that play important roles in temperate forest ecosystems are root entry via N deposition to the soil surface and direct uptake through the stomata in leaves. Although plants may meet their N demand exclusively by root uptake of nitrate ( $\text{NO}_3^-$ ), ammonium ( $\text{NH}_4^+$ ), and dissolved organic N from the soil, many species are capable of incorporating atmospheric reactive N compounds, including  $\text{NO}_y$  and  $\text{NH}_x$ , directly through the leaves and assimilating this N into plant metabolism (Hereid & Monson 2001, Sparks *et al.* 2001, Vallano & Sparks 2007, Vallano & Sparks 2008). If the foliar N uptake pathway has a differential influence on plant metabolism compared to increased soil N availability, then understanding the source N partitioning between these two pathways will be necessary for predicting how forests will respond to the increasing atmospheric N deposition (Sparks 2009 and references therein).

Stable N isotope techniques are useful for estimating the amount of atmospheric reactive N in a system and identifying the uptake pathway of N sources into plant metabolism (Evans 2001, Handley & Raven 1992, Robinson 2001). Several studies have examined the link between N isotopic composition ( $\delta^{15}\text{N}$ ) in plant and soil material to identify the correlations between foliar  $\delta^{15}\text{N}$  patterns and anthropogenic N inputs to temperate forest ecosystems (Amundson *et al.* 2003, Emmett *et al.* 1998, Garten & Vanmiegroet 1994, Hogberg 1997, Kahmen *et al.* 2008, Templer *et al.* 2007). Such measurements serve as practical tool for investigating stable isotope patterns across regional scales and providing an integrated measure of N cycling within a system (Pardo *et al.* 2007, Pardo *et al.* 2006). Further, foliar  $\delta^{15}\text{N}$  values that reflect the  $\delta^{15}\text{N}$  of distinct N sources have the potential to provide important information regarding the causal relationships between plant uptake, assimilation, and allocation of N from natural and human-derived N sources. However, few studies have successfully used this tool to examine the partitioning of N between the soil and foliar uptake pathways, and in particular, the magnitude of foliar N uptake of atmospheric reactive N deposition.

Despite the potential to use foliar  $\delta^{15}\text{N}$  as an indicator of plant N uptake patterns and N cycling, variation in the  $\delta^{15}\text{N}$  of N sources, fractionation due to mycorrhizal associations, and an incomplete understanding of mass dependent fractionation within plants has made it extremely challenging to accurately interpret foliar  $\delta^{15}\text{N}$  (Dawson *et al.* 2002, Evans 2001, Handley *et al.* 1998). Additionally, differences in the capacity for direct foliar uptake among plant species may also significantly impact the observed foliar  $\delta^{15}\text{N}$  patterns. To successfully detect and estimate the relative role of the foliar

uptake pathway, greater understanding of the integrated effects of these processes on foliar  $\delta^{15}\text{N}$  patterns is necessary. Assuming that foliar  $\delta^{15}\text{N}$  values represent the integration of factors such as soil  $\delta^{15}\text{N}$ , direct foliar uptake of atmospheric reactive N deposition, and fractionation due to mycorrhizal associations during plant N assimilation, we sought to determine whether natural abundance soil and foliar  $\delta^{15}\text{N}$  measurements can serve as reliable indicators of atmospheric reactive N inputs, specifically via direct foliar N uptake, in temperate forest ecosystems.

In this study, we attempted to partition and estimate the relative importance of soil  $\delta^{15}\text{N}$ , mycorrhizal associations, and foliar N uptake on foliar  $\delta^{15}\text{N}$  patterns on several hardwood tree species along a gradient of increasing atmospheric  $\text{NO}_2$  concentration in New York State. Assuming that within-plant processes (e.g. translocation and assimilation) were similar within a species, we addressed these relationships by using a linked approach: (1) we estimated the influence of mycorrhizal fractionation on foliar  $\delta^{15}\text{N}$  patterns in seedlings using a potted plant mesocosm experiment where the influence of mycorrhizal fungi was eliminated; (2) we conducted intensive field measurements of foliar and soil  $\delta^{15}\text{N}$  along the same timescale in adult stands of trees and assessed the predictive ability of soil  $\delta^{15}\text{N}$  versus atmospheric  $\text{NO}_2$  concentration on foliar  $\delta^{15}\text{N}$  patterns using multiple regression models. Additionally, we estimated the influence of stomatal conductance and photosynthetic capacity by measuring foliar  $\delta^{13}\text{C}$  patterns among species. Using this dual approach strategy, we sought to determine whether these measurements could be used to estimate the predictive role of these factors on variation in foliar  $\delta^{15}\text{N}$  patterns and assess the potential impact of foliar uptake of reactive N

deposition on temperate forest ecosystems. Alternatively, differences in soil N processing and within-plant N fractionations among species could preclude the interpretation of foliar  $\delta^{15}\text{N}$  patterns and the detection of foliar N uptake in these systems.

## Materials and Methods

### *Site descriptions*

The study was conducted at seven sites in eastern New York State across a north-south gradient of increasing N deposition (Fig. 4.1). Each site contained a mixed-hardwood forest comprised of clusters of adult stands of target tree species (Table 4.1). The sites included stands dominated by one of the

#### Site Locations

1. Huntington Wildlife Forest (HWF)
2. Cary Institute of Ecosystem Studies (IES)
3. Fahnestock State Park (FSP)
4. Mianus River Gorge (MRG)
5. Louis Calder Center (LCC)
6. Inwood Hill Park (IHP)
7. New York Botanical Garden (NYBG)

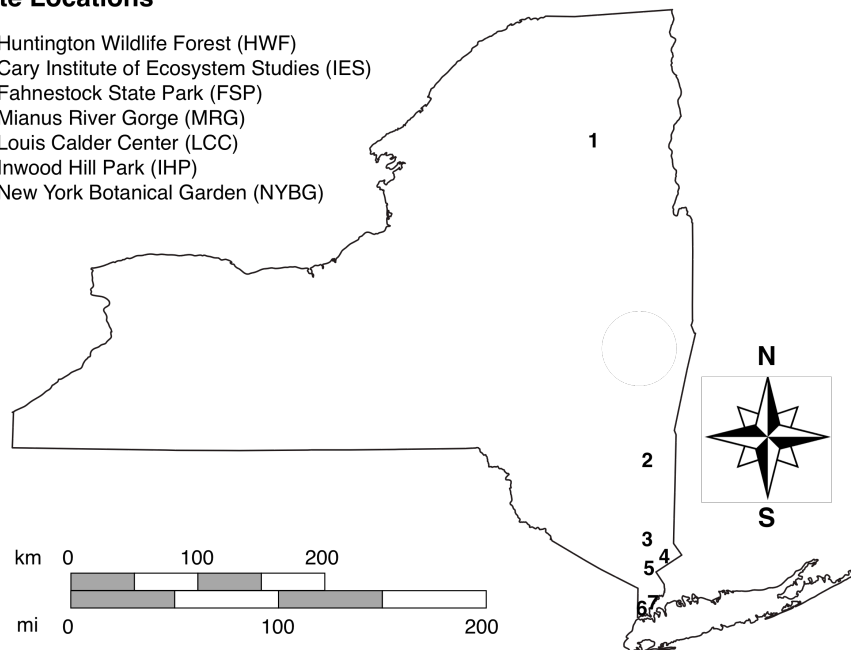


Figure 4.1. Site locations in New York State.

Table 4.1. Site descriptions.

Location	Longitude	Latitude	Ambient NO <sub>2</sub> (ppb)	Species sampled
Huntington Wildlife Forest, Newcomb, NY	-74°13'	43°57'	3.36	<i>Acer rubrum</i> , <i>Fagus grandifolia</i> , <i>Quercus rubra</i>
Cary Institute of Ecosystem Studies, Millbrook, NY	-73°45'	41°47'	5.63	<i>Acer rubrum</i> , <i>Betula lenta</i> , <i>Fagus</i> <i>grandifolia</i> , <i>Quercus rubra</i>
Fahnestock State Park, Carmel, NY	-73°49'	41°27'	26.62	<i>Acer rubrum</i> , <i>Betula lenta</i> , <i>Fagus</i> <i>grandifolia</i> , <i>Quercus rubra</i>
Mianus River Gorge, Bedford, NY	-73°37'	41°10'	12.46	<i>Acer rubrum</i> , <i>Betula lenta</i> , <i>Fagus</i> <i>grandifolia</i> , <i>Quercus rubra</i>
Louis Calder Center, Armonk, NY	-73°44'	41°07'	16.96	<i>Acer rubrum</i> , <i>Betula lenta</i> , <i>Fagus</i> <i>grandifolia</i> , <i>Quercus rubra</i>
Inwood Hill Park, NYC, NY	-73°55'	40°52'	34.19	<i>Acer rubrum</i> , <i>Betula lenta</i> , <i>Fagus</i> <i>grandifolia</i> , <i>Quercus rubra</i>
The New York Botanical Garden, NYC, NY	-73°52'	40°51'	36.76	<i>Acer rubrum</i> , <i>Betula lenta</i> , <i>Fagus</i> <i>grandifolia</i> , <i>Quercus rubra</i>

following tree species: American beech (*Fagus grandifolia*), red oak (*Quercus rubra*), red maple (*Acer rubrum*), and black birch (*Betula lenta*). A similar transect was established to measure the effects of urbanization on forest ecosystems (Pouyat et al. 1995) and to determine patterns of bulk N deposition, oak forest throughfall, and particulate dust deposition (Lovett et al. 2000). All sites are in close proximity to regional pollutant monitoring networks (e.g. the National Atmospheric Deposition Program/National Trends Network and the Clean Air Status and Trends Network) so that total N deposition (wet + dry inorganic N) was monitored and estimated at each site. Passive air samplers (Ogawa and Co. USA Inc., Pompano Beach, FL, USA) were installed and measured ambient atmospheric nitrogen dioxide (NO<sub>2</sub>) concentrations at each site from June 3, to September 17, 2007 (Table 4.1). Samplers were placed approximately 1 m above ground level, and when possible, installed adjacent to a potted plant mesocosm system. Samplers were housed in a shelter to minimize effects from precipitation and duplicate

samples were collected at each site ( $n = 2$ ). After exposure, samplers were immediately stored at 4°C and analyzed for ambient NO<sub>2</sub> concentrations using an Astoria Pacific series 300 autoanalyzer (Astoria-Pacific Inc., Clackamas, OR, USA).

### *Potted Plant Mesocosm Study*

Red maple (*A. rubrum*) seedlings, approximately 0.3-0.4 m tall were obtained bare root from Pinelands Nursery and Supply (Columbus, NJ, USA). Seedlings were selected for uniformity of height, transplanted into 12-L pots containing a mixture of perlite and vermiculite (Sun-Gro Horticulture, Bellevue, WA, USA), and grown in a climate-controlled growth chamber (EGC, Chagrin Falls, OH, USA) at day and night temperatures of 27 and 21 °C, respectively, under moderate light (600  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) and a 16-h photoperiod. Seedlings were watered daily to saturation for 12 weeks and fertilized regularly with a modified Hoagland's solution (Smart & Bloom 1993) containing NO<sub>3</sub><sup>-</sup> with a known  $\delta^{15}\text{N}$ . Measurements of seedling size (height and stem diameter at 2.5 cm above the root collar),  $\delta^{15}\text{N}$ ,  $\delta^{13}\text{C}$  and N and C content were made on a subset of seedlings prior to the start of the experiment to provide a baseline for experimental plants ( $n = 20$ ).

Potted plant mesocosm systems were established at five of the sites (The New York Botanical Garden, Louis Calder Center, Mianus River Gorge, Cary Institute of Ecosystem Studies, and Huntington Wildlife Forest) in June 2007 and maintained through September 2007. Mesocosm systems were established in open fields exposed to full sun. Each system was approximately



2.5 x 4.0 m and enclosed by fencing to eliminate browsing by deer and other small mammals. At each site, seedlings ( $n = 10$ ) were randomly placed in holes to the depth of the pot and filled in with natural soil. All holes were dug 50% beyond the depth of the pot and filled with gravel and sand to allow for ample drainage. A 15 cm diameter hole was cut from the bottom of each pot to further allow for maximum drainage. Seedlings were drip irrigated to match natural precipitation patterns throughout the experiment using a 95-L barrel as a reservoir and an automatic drip irrigation system (Raindrip Inc., Fresno, CA, USA). To minimize any effects of atmospheric reactive N deposited to the soil surface, pots were covered with an opaque plastic shield (Ithaca Plastics, Ithaca, NY, USA) suspended around the base of each seedling and open on all sides to allow for adequate airflow and soil evaporation. This planting strategy matched the rooting zone temperature to the site during the growing season, allowed for irrigation, and minimized soil N from the environment entering each pot by lateral flow.

The experimental system was composed of two treatments: 'controls' with natural levels of root infection by mycorrhizae and 'fungicide-treated' plants where the fungicide Rovral (10% w/w iprodione, Bayer CropScience, Research Triangle Park, NC, USA) was applied to eliminate the influence of mycorrhizae on foliar  $\delta^{15}\text{N}$ . Rovral was applied every 3 weeks at rates of 2.0 g/m<sup>2</sup> of soil surface in 500 ml of deionized water to half of the seedlings ( $n = 5$ ) to reduce mycorrhizal colonization of roots (Gange et al. 1990, St Clair & Lynch 2005). The fungicide treatments were applied according to the methods of Ganade and Brown (1997). Rovral has been shown to be effective in reducing infection by mycorrhizal fungi in plant roots for a wide variety of plant species (Gange et

al. 1990, Gange et al. 1993, St Clair & Lynch 2005). For many of the previously tested species, the fungicide did not appear to directly affect the plant or seed performance (Gange et al. 1992). Further, no negative side effects were detected on a range of soil invertebrates (Ganade & Brown 1997, Gange et al. 1993). To assess the bulk soil  $\delta^{15}\text{N}$ , soil cores were taken every 3 weeks between June 6 and September 12 2007 from each pot at a depth of 0-10 cm using a soil corer.

Seedlings were harvested in September 2007 (or prior to the experiment for baseline seedlings) and separated into leaf, shoot, and root tissues. Shoot length and leaf number were measured for each individual and leaf area was estimated for a subset of sample ( $n = 3$ ) using a leaf area meter (LI-3100 Area Meter, LI-COR, Inc., Lincoln, NE, USA).

### *Field Sampling*

Leaf and soil samples were collected every 3 weeks between June 6 and September 12 2007 from all seven sites (Fig. 4.1, Table 4.1) from June 2007 through September 2007. Leaf samples from 3-4 dominant species were collected using a wrist rocket (Falcon 2, Stunning Sales, Garland, TX, USA) from 5 randomly selected trees with a diameter breast height  $\geq 15$  cm at each site (Table 4.1). To assess the bulk soil  $\delta^{15}\text{N}$ , soil cores were taken at every 3 weeks from the base of each sampled tree at a depth of 0-10 cm using a soil corer.

### *Stable Isotopic Analysis*

Plant tissue samples from both the potted plant mesocosm study and field sampling were rinsed with deionized water to remove any reactive N deposited to the leaf surface and dried at 55 °C for 72-h. The dried plant tissue samples were weighed, ground to a fine powder with a mortar and pestle, and sub-samples of 2.55-3.15 mg were weighed for isotopic analysis using a microbalance (Model 4504MP8; Sartorius Corp. Edgewood, NY, USA). Bulk soil samples were dried at 65°C for 48-h, sieved using a 2-mm mesh screen, ground to a fine powder using a mortar and pestle, and stored at 4°C until the time of analysis. Bulk soil and leaf N and C concentration were measured using a CHN elemental analyzer (Model Carlo Erba NC2500; Thermo Finnigan, San Jose, CA, USA).  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  were measured using a continuous flow isotope ratio mass spectrometer (Model Delta V; Thermo Finnigan, San Jose, CA, USA). All analyses were conducted at the Stable Isotope Laboratory at Cornell University, Ithaca, NY.

### *Statistical Analysis*

We examined differences in  $\Delta^{15}\text{N}$  (the difference between foliar and soil  $\delta^{15}\text{N}$ ),  $\delta^{15}\text{N}$ ,  $\delta^{13}\text{C}$  and %N values among samples using two-way analyses of variance (ANOVA), with mycorrhizal treatment and  $\text{NO}_2$  concentration as the main effects. Tukey's HSD procedure was used to make pairwise comparisons ( $\alpha = 0.05$ ) within and between field sites. We used single linear regression models to compare potential relationships among  $\Delta^{15}\text{N}$ ,  $\delta^{15}\text{N}$ ,  $\delta^{13}\text{C}$ , %N, and  $\text{NO}_2$  concentration for the potted plant and field data. Multiple linear regression

models were constructed using soil  $\delta^{15}\text{N}$  and  $\text{NO}_2$  concentration to explain variation in foliar  $\delta^{15}\text{N}$ . The  $r^2$  selection method was used to determine the combination of predictors that resulted in the best multiple regression models explaining foliar  $\delta^{15}\text{N}$ . Significance levels for all tests were  $P \leq 0.05$ . Statistical analyses were performed using SAS JMP software (Version 7.0, 2007).

## Results

### *Potted plant mesocosm experiment*

We observed a positive relationship between foliar  $\delta^{15}\text{N}$  and increasing  $\text{NO}_2$  concentration in red maple seedlings with mycorrhizal associations ( $r^2 = 0.28$   $P = 0.01$ ), although there was no trend in plants without mycorrhizal associations (Fig. 4.2a). There were significant differences in foliar  $\delta^{15}\text{N}$  between

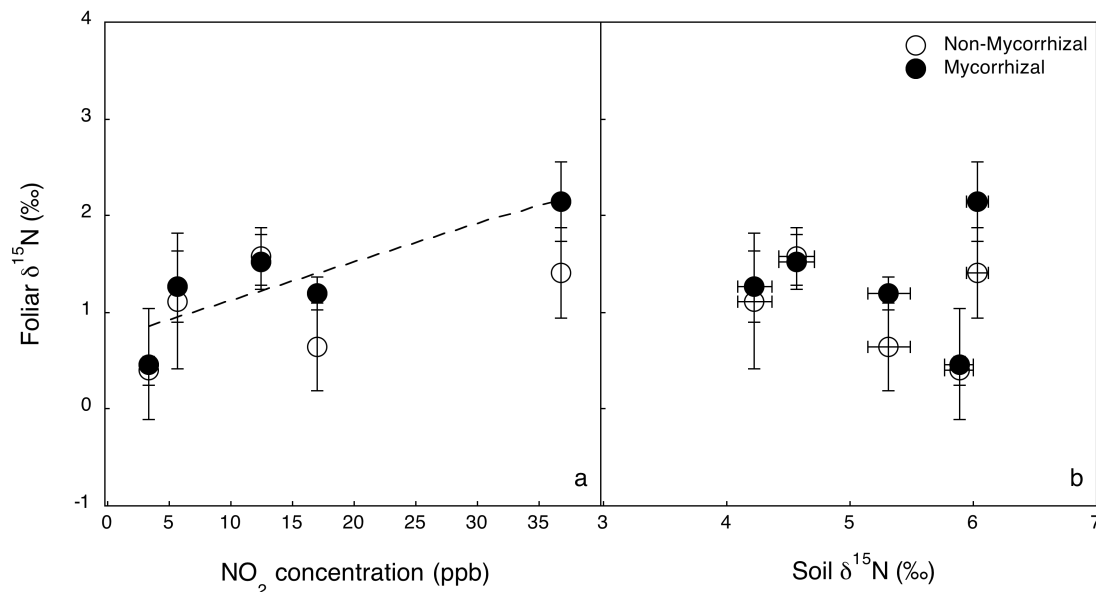


Figure 4.2. Relationships between ambient atmospheric nitrogen dioxide concentration (a) and bulk soil  $\delta^{15}\text{N}$  (b) with foliar  $\delta^{15}\text{N}$  in red maple seedlings without mycorrhizal associations (open circles) and with mycorrhizal associations (closed circles). Data are means  $\pm 1$  standard error ( $n = 5$ ).

mycorrhizal and non-mycorrhizal plants ( $P < 0.01$ ) when considered across sites along the  $\text{NO}_2$  concentration gradient. When the influence of mycorrhizae was removed, foliar  $\delta^{15}\text{N}$  values were altered 0.03 – 1.01‰ depending on the site location. We found no significant correlation between foliar and soil  $\delta^{15}\text{N}$  in red maple, regardless of mycorrhizal associations (Fig. 4.2b). We estimated the influence of mycorrhizal fractionation on foliar  $\delta^{15}\text{N}$  values by calculating the difference in foliar  $\delta^{15}\text{N}$  between the mycorrhizal and non-mycorrhizal individuals for each site along the atmospheric  $\text{NO}_2$  concentration gradient. Defined as the mycorrhizal fractionation, the correction factor had a range of –0.055 to 0.741 ‰ across sites (data not shown).

There was no trend between foliar  $\delta^{13}\text{C}$  and increasing  $\text{NO}_2$  concentration in red maple, regardless of the mycorrhizal treatment (Fig. 4.3). Foliar N content was negatively correlated with increasing  $\text{NO}_2$  concentration in red maple with mycorrhizal associations ( $r^2 = 0.30$ ,  $P < 0.01$ ) and without mycorrhizal associations ( $r^2 = 0.45$ ,  $P = 0.0004$ ). However, this trend was driven completely by a single site location and is likely not strongly correlated with increasing  $\text{NO}_2$  concentration along the gradient. There were no differences in foliar N content between mycorrhizal and non-mycorrhizal treatments when considered across sites along the atmospheric  $\text{NO}_2$  concentration gradient.

### *Field Sampling*

There were significant trends between  $\Delta^{15}\text{N}$  (the absolute difference between foliar and soil  $\delta^{15}\text{N}$ ) and both soil  $\delta^{15}\text{N}$  and increasing  $\text{NO}_2$  concentration on foliar  $\delta^{15}\text{N}$  patterns in tree species. The  $\Delta^{15}\text{N}$  value for all species showed a

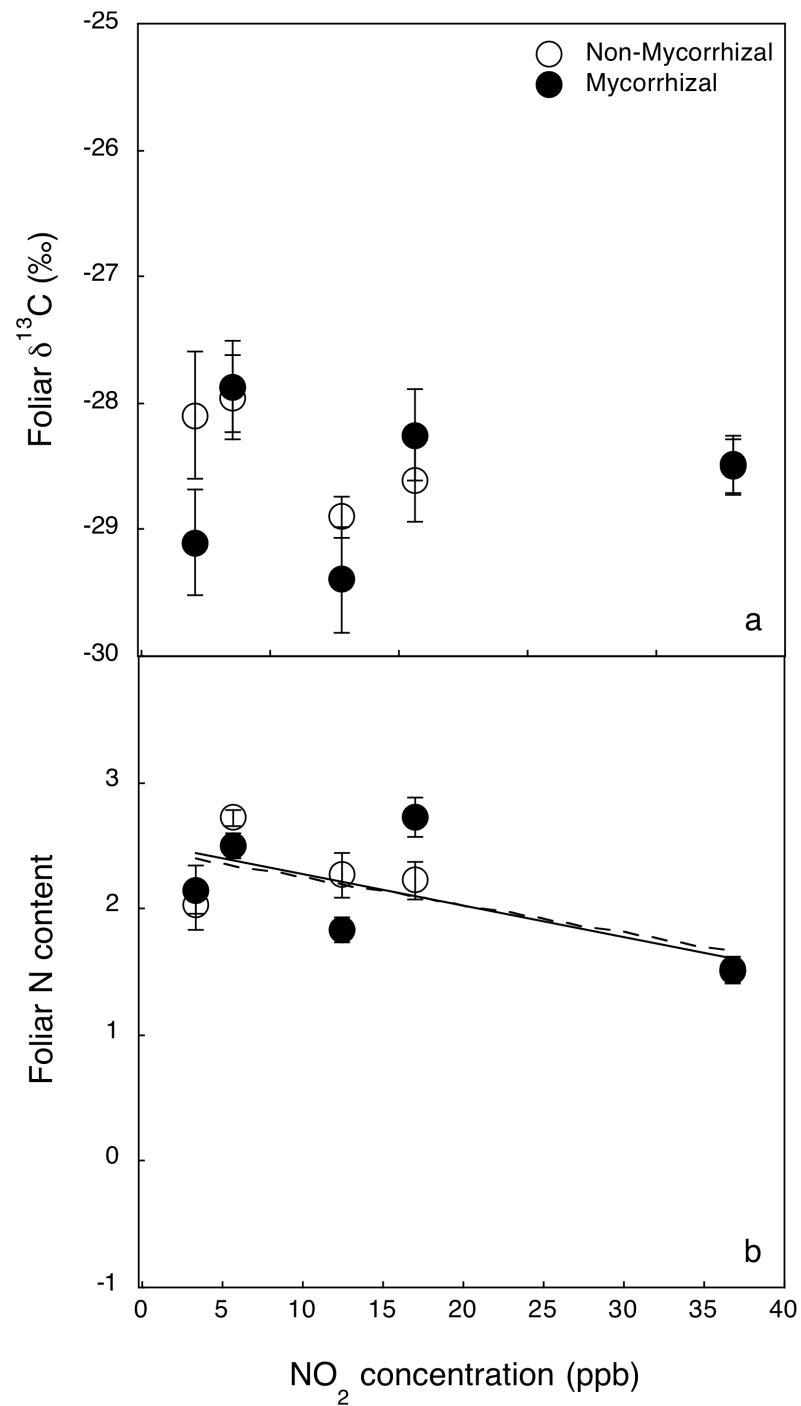


Figure 4.3. Relationship between ambient atmospheric nitrogen dioxide concentration and (a) foliar  $\delta^{13}\text{C}$  in red maple seedlings without mycorrhizal associations (open circles) and with mycorrhizal associations (closed circles) and (b) foliar nitrogen content in seedlings without mycorrhizal associations (open squares) and with mycorrhizal associations (closed squares). Data are means  $\pm 1$  standard error ( $n = 5$ ).

negative relationship with increasing  $\text{NO}_2$  concentration (Fig. 4.4). Black birch and American beech showed a negative correlation between  $\Delta^{15}\text{N}$  and increasing  $\text{NO}_2$  concentration ( $P = 0.03$  and  $P = 0.02$ , respectively). Although not significant at  $\alpha = 0.05$ , we observed a negative trend between  $\Delta^{15}\text{N}$  and increasing  $\text{NO}_2$  concentration in red oak ( $P = 0.07$ ). There was not a significant correlation between  $\Delta^{15}\text{N}$  and increasing  $\text{NO}_2$  concentration in red maple.

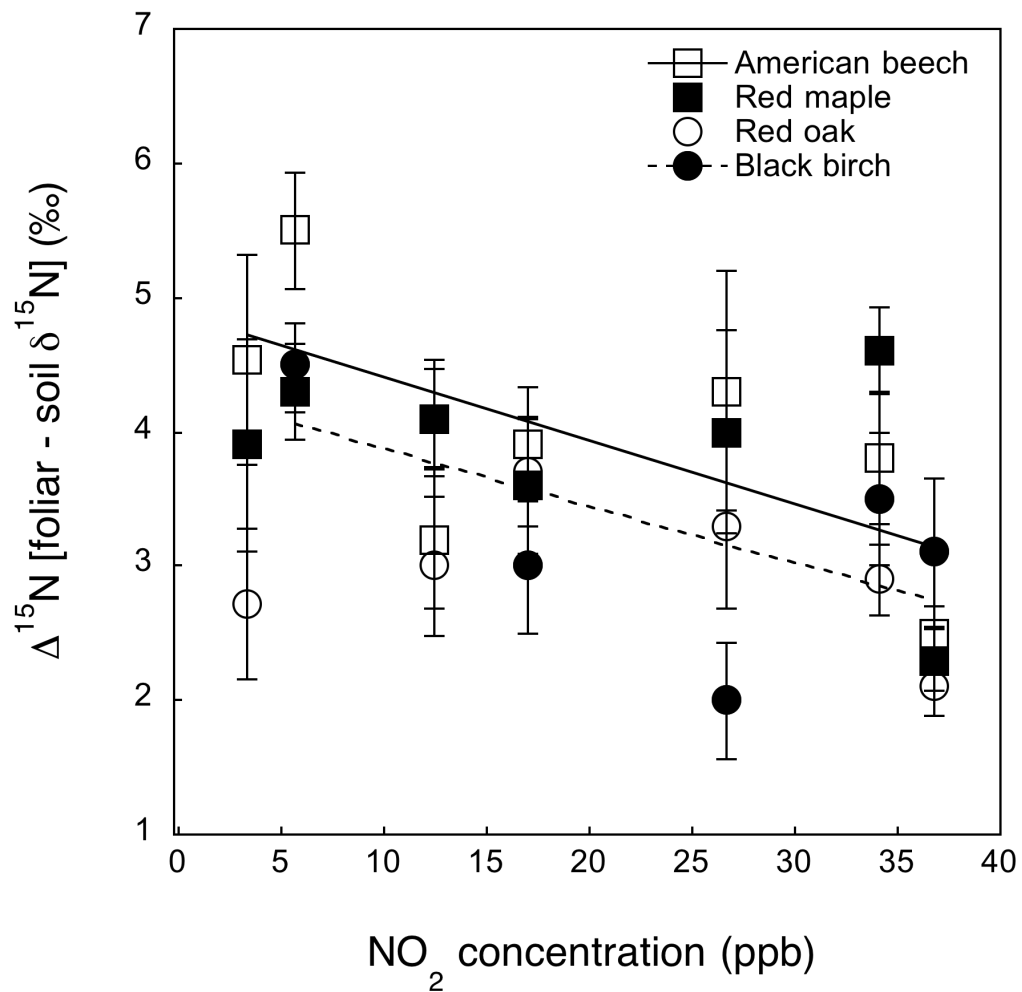


Figure 4.4. Relationship between  $\Delta\delta^{15}\text{N}$  (the absolute difference between soil  $\delta^{15}\text{N}$  and foliar  $\delta^{15}\text{N}$ ) and ambient atmospheric nitrogen dioxide concentration in adult trees. Species include American beech (open squares), red maple (closed squares), red oak (open circles) and black birch (closed circles). Data are means  $\pm 1$  standard error ( $n = 20$ ).

Single linear regressions were used to determine the influence of soil  $\delta^{15}\text{N}$  and increasing  $\text{NO}_2$  concentration on foliar  $\delta^{15}\text{N}$  patterns in tree species. In general, we found significant correlations between foliar  $\delta^{15}\text{N}$  and both soil  $\delta^{15}\text{N}$  and increasing  $\text{NO}_2$  concentration when tested singly (Fig. 4.5). There were significant and positive relationships between foliar and soil  $\delta^{15}\text{N}$  values in the following species: American beech (Fig. 4.5a), red maple (Fig. 4.5c), red oak (Fig. 4.5e), and black birch (Fig. 4.5g). We also found that foliar  $\delta^{15}\text{N}$  was negatively correlated with increasing  $\text{NO}_2$  concentration in the following species: American beech (Fig. 4.5b), red maple (Fig. 4.5d), and red oak (Fig. 4.5f). However, there was not a significant relationship between foliar  $\delta^{15}\text{N}$  and increasing  $\text{NO}_2$  concentration in black birch.

Patterns in foliar  $\delta^{15}\text{N}$  were best explained by a multiple regression model containing both soil  $\delta^{15}\text{N}$  and the average  $\text{NO}_2$  concentration (in ppb) at each site as predictors (Fig. 4.6). Multiple linear regression models containing both factors explained 40%, 56%, 47%, and 43% in American beech, red maple, red oak, and black birch, respectively. Partial residual plots for each predictor were generated to determine how much of the variation in foliar  $\delta^{15}\text{N}$  was explained by increasing  $\text{NO}_2$  concentration after accounting for the effects of soil  $\delta^{15}\text{N}$ , the factor with the strongest relationship to foliar  $\delta^{15}\text{N}$ . After accounting for soil  $\delta^{15}\text{N}$ , increasing  $\text{NO}_2$  concentration explained 0%, 69%, 23%, and 45% of the residual variation in foliar  $\delta^{15}\text{N}$  remaining in American beech, red maple, red oak, and black birch, respectively.

Applying the mycorrhizal correction factor calculated using the potted plant mesocosm foliar  $\delta^{15}\text{N}$  data did not alter the field relationship between foliar



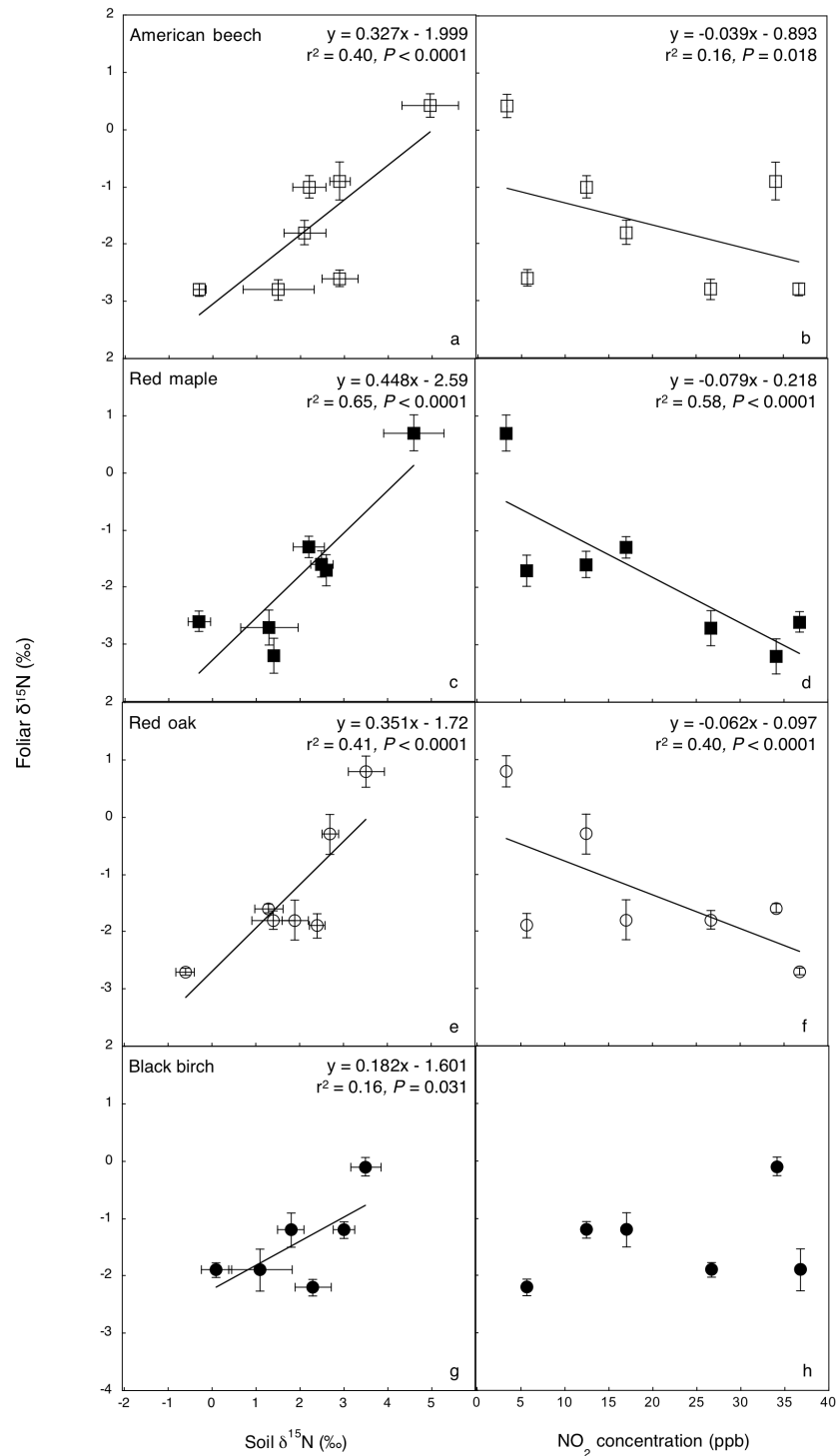


Figure 4.5. Relationship between foliar  $\delta^{15}\text{N}$  and bulk soil  $\delta^{15}\text{N}$  (a, c, e, g) and ambient atmospheric nitrogen dioxide concentration (b, d, f, h) in adult trees. Species include American beech (open squares), red maple (closed squares), red oak (open circles) and black birch (closed circles). Data are means  $\pm$  1 standard error ( $n = 20$ ).

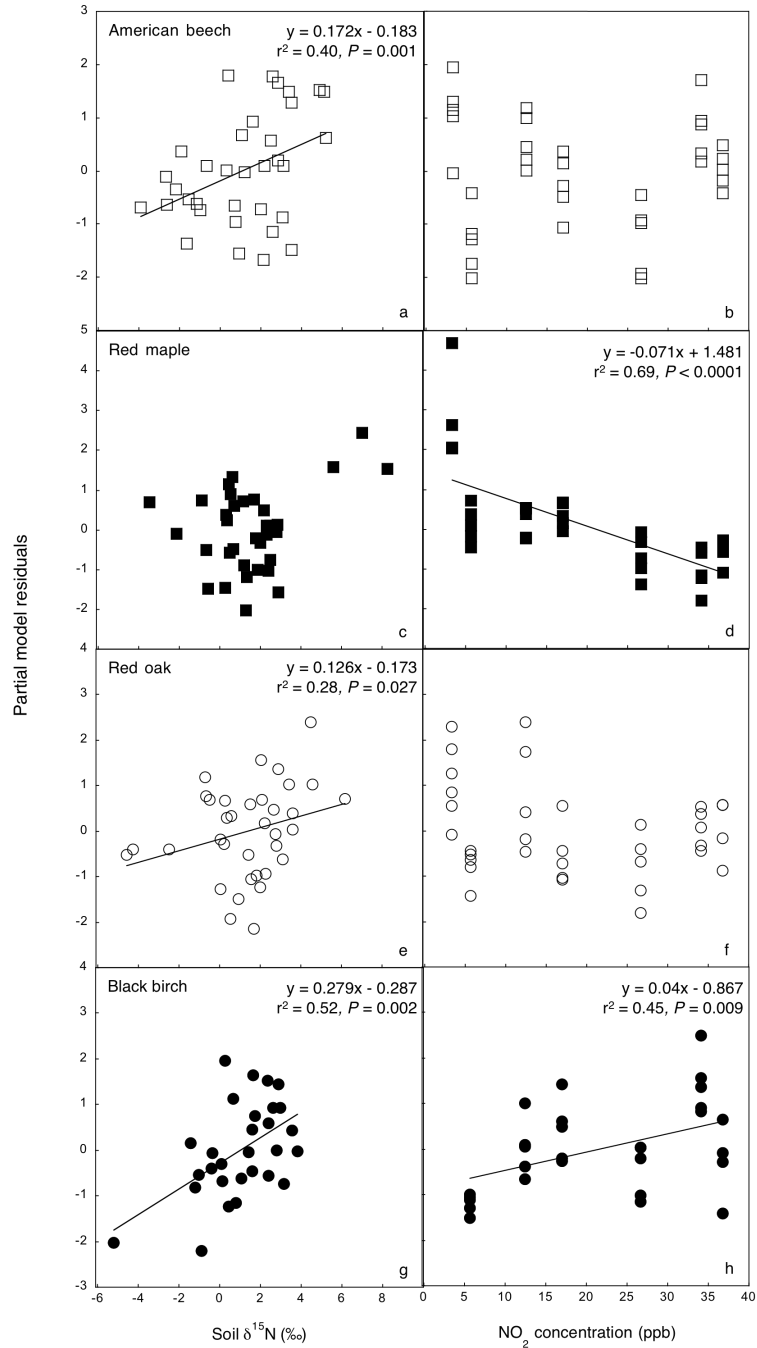


Figure 4.6. Partial residual plots for the predictor variables included in the multiple regression model explaining foliar  $\delta^{15}\text{N}$ . The correlation of bulk soil  $\delta^{15}\text{N}$  and the residuals of the model describing foliar  $\delta^{15}\text{N}$  with  $\text{NO}_2$  concentration (a, c, e, g) in tree species. The correlation of  $\text{NO}_2$  concentration and the residuals of the model describing foliar  $\delta^{15}\text{N}$  with bulk soil  $\delta^{15}\text{N}$  (b, d, f, h) in tree species. Species include American beech (open squares), red maple (closed squares), red oak (open circles) and black birch (closed circles). Data are means  $\pm$  1 standard error ( $n = 20$ ).

$\delta^{15}\text{N}$ , soil  $\delta^{15}\text{N}$  and  $\text{NO}_2$  concentration in red maple (Fig. 4.7). When this simplistic mycorrhizal correction factor was applied, there was no significant difference in the relationship between foliar  $\delta^{15}\text{N}$  and  $\text{NO}_2$  concentration in corrected ( $r^2 = 0.54$ ,  $P < 0.0001$ ) and uncorrected data ( $r^2 = 0.52$ ,  $P < 0.0001$ , Fig. 4.7a). Similarly, there was not a difference in the relationship between foliar and soil  $\delta^{15}\text{N}$  between corrected ( $r^2 = 0.97$ ,  $P < 0.0001$ ) and un-corrected ( $r^2 = 0.87$ ,  $P < 0.0001$ ) data (Fig. 4.7b).

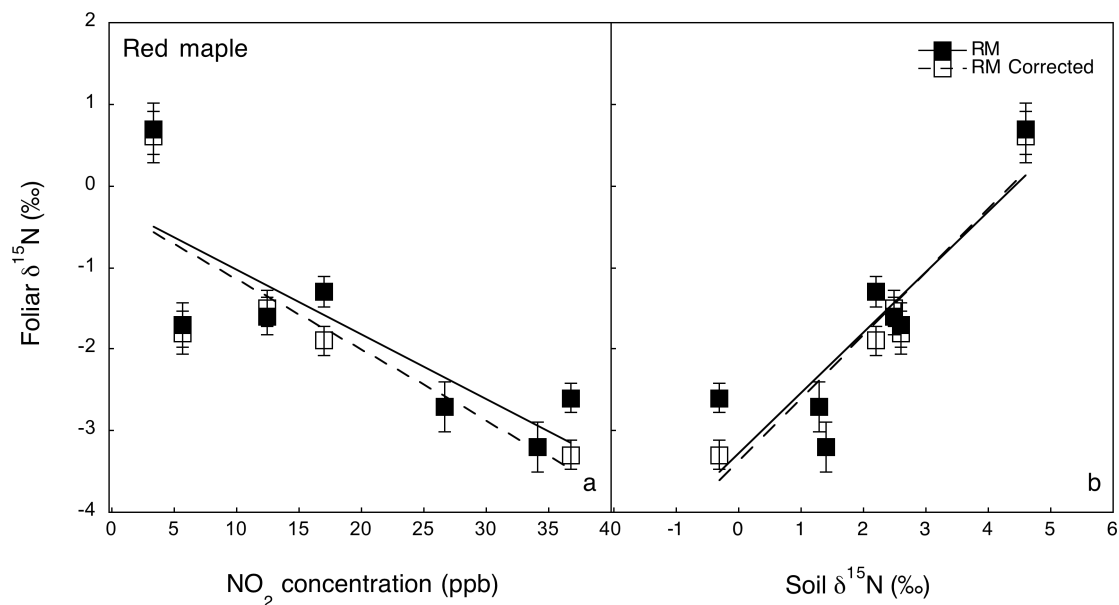


Figure 4.7. Relationship between foliar  $\delta^{15}\text{N}$  and (a) ambient atmospheric nitrogen dioxide concentration and (b) bulk soil  $\delta^{15}\text{N}$  in red maple trees. Data represent original foliar  $\delta^{15}\text{N}$  (open squares) and foliar  $\delta^{15}\text{N}$  corrected for mycorrhizal fractionation (closed squares). Data are means  $\pm 1$  standard error ( $n = 20$ ).

In general, we observed no relationship between foliar  $\delta^{13}\text{C}$  and increasing  $\text{NO}_2$  concentration in most species, with the exception of a negative trend between foliar  $\delta^{13}\text{C}$  and increasing  $\text{NO}_2$  concentration in red maple ( $r^2 = 0.19$ ,  $P < 0.01$ , Fig. 4.8a). Likewise, we found no relationship between foliar N content and increasing  $\text{NO}_2$  concentration in most species (Fig. 4.8b).

However, there was a negative correlation between foliar N content and increasing  $\text{NO}_2$  concentration in red maple ( $r^2 = 0.21$ ,  $P = 0.006$ ).

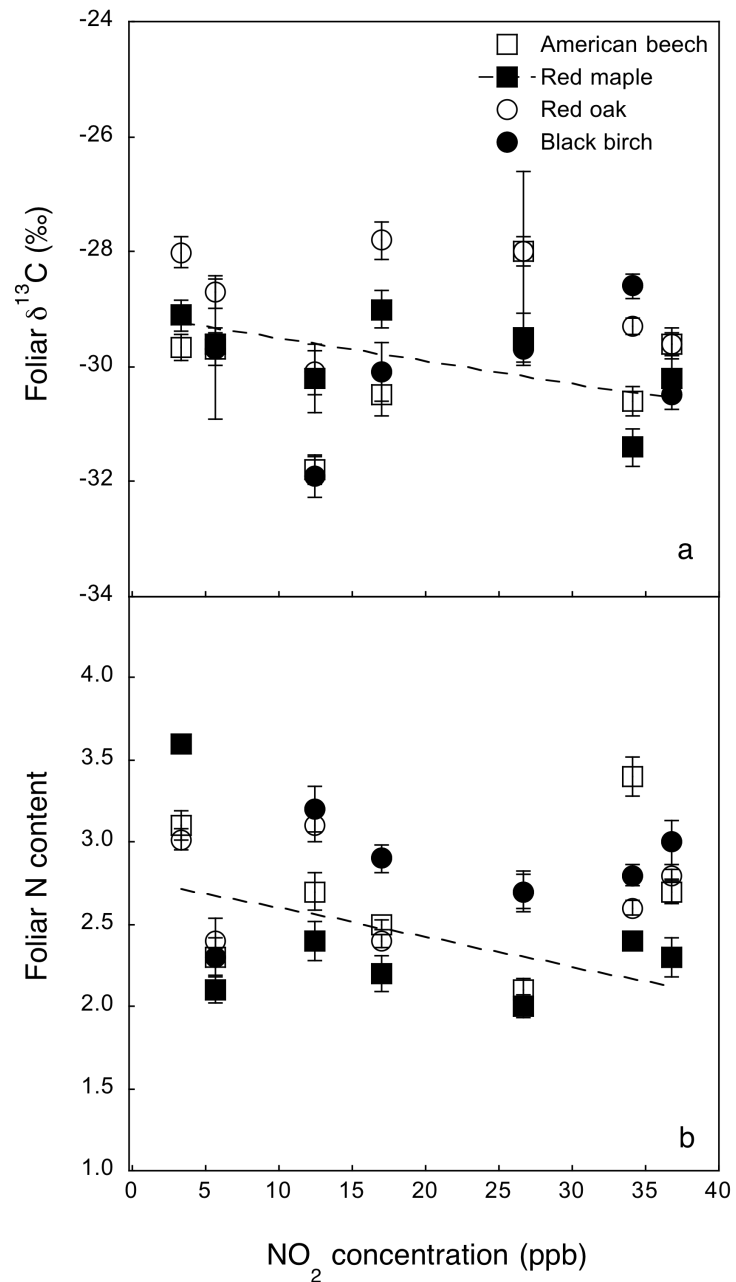


Figure 4.8. Relationship between ambient atmospheric nitrogen dioxide concentration and (a) foliar  $\delta^{13}\text{C}$  and (b) foliar nitrogen content in adult trees. Species include American beech (open squares), red maple (closed squares), red oak (open circles) and black birch (closed circles). Data are means  $\pm$  1 standard error ( $n = 20$ ).

## Discussion

We found significant trends in natural abundance foliar  $\delta^{15}\text{N}$  values across sites along a transect of increasing atmospheric  $\text{NO}_2$  concentration in both red maple seedlings and in adult trees species of American beech, red maple, red oak, and black birch (Figs. 4.2, 4.5). This range of  $-4$ - $2\text{‰}$  was similar to previous values recorded in other temperate forest studies (Nadelhoffer & Fry 1994, Pardo *et al.* 2007, Pardo *et al.* 2006, Templer *et al.* 2007). Overall, we found strong patterns of decreasing foliar  $\delta^{15}\text{N}$  values across the following species: Red maple > red oak > American beech > black birch. These patterns in foliar  $\delta^{15}\text{N}$  observed in adult tree species may be potentially linked to lower nitrification rates in the soil N pool (Pardo *et al.* 2007). Additionally, it is likely these patterns are driven by systematic variation among species, possibly due to a suite of differences in rooting depth, availability of  $\text{NH}_4^+$  versus  $\text{NO}_3^-$  in the soil N pool, phenology, local nitrification rates, and mycorrhizal associations (Nadelhoffer *et al.* 1999).

Variation in mycorrhizal fractionation among species is thought to be a controlling driver of foliar  $\delta^{15}\text{N}$  patterns, although our results did not show support for such a relationship in red maple seedlings. For instance, it has been shown that ecto- or ericoid mycorrhizal species such as red oak typically show a greater degree of isotopic fractionation associated with root N uptake from the soil N pool (Hobbie & Hobbie 2008). However, red maple is a vesicular arbuscular mycorrhizal species and such species are assumed to discriminate only marginally against  $^{15}\text{N}$  during uptake (Handley & Raven 1992), which could explain the observed patterns found in this study. Further,

applying the mycorrhizal correction factor calculated using the potted plant mesocosm foliar  $\delta^{15}\text{N}$  data did not alter the field relationship between foliar  $\delta^{15}\text{N}$ , soil  $\delta^{15}\text{N}$  and  $\text{NO}_2$  concentration in red maple, suggesting that mycorrhizal fractionation did not strongly influence foliar  $\delta^{15}\text{N}$  patterns in adult stands of red maple (Fig. 4.7).

In general, we did not find significant correlations between either foliar  $\delta^{13}\text{C}$  or foliar N content and increasing  $\text{NO}_2$  concentration across sites along the transect (Figs. 4.3, 4.8), suggesting that variation in integrated seasonal stomatal conductance was similar across sites along the transect. Additionally, these results indicate that it is unlikely differential rates of atmospheric  $\text{NO}_2$  flux into leaves were likely playing a role in driving the observed foliar  $\delta^{15}\text{N}$  patterns.

We used patterns in  $\Delta\delta^{15}\text{N}$ , or the absolute deviation of foliar  $\delta^{15}\text{N}$  from soil  $\delta^{15}\text{N}$ , to infer the sensitivity of plant species at each of the sites to increasing rates of human-derived reactive N deposition (Fig. 4.4). Consistent with previous studies, foliar  $\delta^{15}\text{N}$  values were depleted relative to surface soil  $\delta^{15}\text{N}$ , usually resulting from mineral soil N uptake which is depleted in  $^{15}\text{N}$  compared to sampled soil surface N or soil organic N (Kahmen *et al.* 2008, Robinson 2001). Regardless of species,  $\Delta\delta^{15}\text{N}$  decreased as the  $\text{NO}_2$  concentration increased across the transect of increasing  $\text{NO}_2$  concentration. These patterns may be interpreted as a shift in the dominant N source of species from mineral soil N sources to atmospheric reactive N sources from human-derived pollution. Variation in the capacity for foliar uptake of atmospheric reactive N among species may be driving the observed species differences in  $\Delta\delta^{15}\text{N}$ .

Additionally, species differences in N uptake preferences may also contribute to the observed variation in  $\Delta^{15}\text{N}$  values across species (Kahmen *et al.* 2008).

We observed strong relationships between foliar  $\delta^{15}\text{N}$ , soil  $\delta^{15}\text{N}$ , and  $\text{NO}_2$  concentration in all species (Fig. 4.5). Overall, we found strong relationships between soil and foliar  $\delta^{15}\text{N}$  values across the following species: Red maple > red oak > American beech > black birch. The general trends among these species are consistent with previous studies that have found a strong relationship between foliar  $\delta^{15}\text{N}$  and relative rates of soil N cycling in temperate forest ecosystems (Emmett *et al.* 1998, Gebauer & Schulze 1991, Hogberg 1990, Pardo *et al.* 2002). We also observed similar relationships between increasing  $\text{NO}_2$  concentration and foliar  $\delta^{15}\text{N}$  values across the following species: Red maple > red oak > American beech > black birch. These results are consistent with previous work that has documented comparable foliar  $\delta^{15}\text{N}$  patterns in red maple and red spruce stands (Pardo *et al.* 2007).

Although results suggest that soil  $\delta^{15}\text{N}$  predominantly drives foliar  $\delta^{15}\text{N}$  patterns in several species, foliar uptake of atmospheric reactive N (as indexed by ambient atmospheric  $\text{NO}_2$  concentration) may also be a strong predictor of variation in foliar  $\delta^{15}\text{N}$  in several species.

The regression model including both soil  $\delta^{15}\text{N}$  and  $\text{NO}_2$  concentration as predictors explained up to 56% of variation in foliar  $\delta^{15}\text{N}$  patterns (Fig. 4.6). When we accounted for the influence of soil  $\delta^{15}\text{N}$  on patterns of foliar  $\delta^{15}\text{N}$  using multiple regression models, increased  $\text{NO}_2$  concentration explained a

large proportion of the remaining variation in foliar  $\delta^{15}\text{N}$  across species. For instance, the strong correlation and explained variance for red maple foliar  $\delta^{15}\text{N}$  with increasing  $\text{NO}_2$  concentration suggests that this species is likely responding directly to foliar uptake of atmospheric  $\text{NO}_2$  or indirectly to physiological and biochemical consequences of increasing atmospheric  $\text{NO}_2$  concentration. Further, the predictive capability of  $\text{NO}_2$  concentration may highlight species-specific differences in the capacity for foliar uptake of atmospheric reactive N deposition.

Despite the strong relationships documented between foliar  $\delta^{15}\text{N}$ , soil  $\delta^{15}\text{N}$ , and  $\text{NO}_2$  concentration (Figs. 4.5, 4.6), the remaining variance not accounted for in our statistical analyses suggests that a possible suite of other factors is influencing foliar  $\delta^{15}\text{N}$  patterns. Soil characteristics, available sources, and N cycling processes likely play a strong role in the overall relationship between soil  $\delta^{15}\text{N}$  and foliar  $\delta^{15}\text{N}$ . Species differences in within-plant fractionation and differences in foliar N uptake rates may also have played a role in the variation in foliar  $\delta^{15}\text{N}$  patterns observed in this study. Within-plant fractionations of N can cause variation of 7-12‰ within individual plants (Evans 2001). Species differences in foliar N uptake of atmospheric reactive N could also influence within-plant variation in foliar  $\delta^{15}\text{N}$  (Morikawa *et al.* 1998). Climate factors such as precipitation, elevation, and land use change have also been shown to exert control on foliar  $\delta^{15}\text{N}$  values (Amundson *et al.* 2003). Further, the capability of plants to bypass mycorrhizal associations when N is more abundant or a shift in mycorrhizal species as N availability increases across sites cannot be definitely ruled out as a driver of the observed foliar  $\delta^{15}\text{N}$  patterns (Hobbie & Hobbie 2008, Hobbie *et al.* 2000).



It is clear that human derived reactive N inputs have impacted rates N isotope cycling in many ecosystems, likely leading to significant changes in the way N is processed and incorporated into ecosystems (Vitousek *et al.* 1997).

However, it is still not well understood how increasing atmospheric reactive N deposition has impacted the foliar  $\delta^{15}\text{N}$  value in ecosystems. In this study, we show that foliar  $\delta^{15}\text{N}$  may be used as an indicator of systematic changes in ecosystem N cycling to characterize the relative influences of various N inputs and transformations available to vegetation, although the complexity of sources, inputs and fractionation still lends itself to great uncertainty in these measurements. Very few, if any studies, have attempted to directly estimate the influence of foliar N uptake on foliar  $\delta^{15}\text{N}$  patterns in trees in temperate forests. Although not conclusive, our results indicate that total canopy uptake, and most likely direct foliar N uptake, of atmospheric reactive N deposition does appear to have an influence on foliar  $\delta^{15}\text{N}$  in several species and can be detected using natural abundance  $\delta^{15}\text{N}$  measurements of plant and soil material. Further work is necessary to clearly identify and quantify the role and  $\delta^{15}\text{N}$  of various plant-available soil N sources such as  $\text{NO}_3^-$ ,  $\text{NH}_4^+$ , and organic N in the soil solution. Additional insight to the contribution of mycorrhizal associations to foliar  $\delta^{15}\text{N}$  patterns is also necessary to adequately assess ecosystem and plant N dynamics on local, regional, and global scales of varying N deposition.

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